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Gamma-Ray Burst

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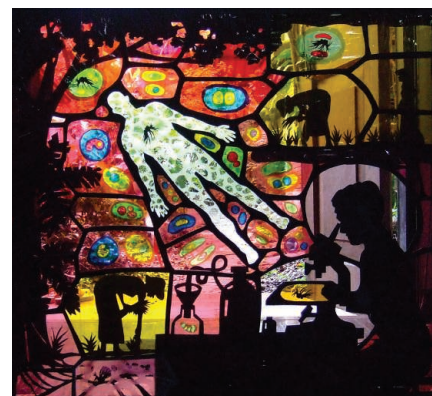
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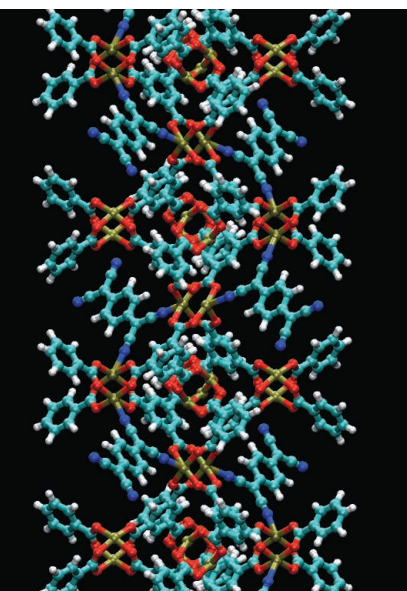
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Artist's conception of gamma-ray burst (GRB) 130427A, one of the brightest and longest-lived GRBs observed to date. GRBs such as this one occur when the core of a massive star runs out of nuclear fuel, collapses, and forms a black hole that drives a powerful jet of plasma traveling close to the speed of light. See pages 34, 38, 42, 48, and 51.

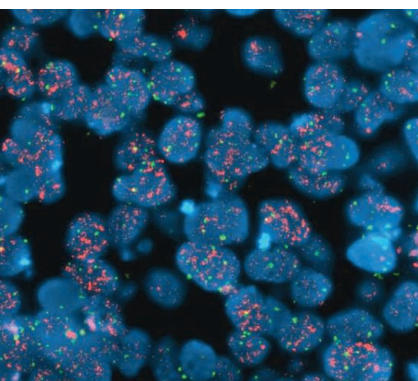
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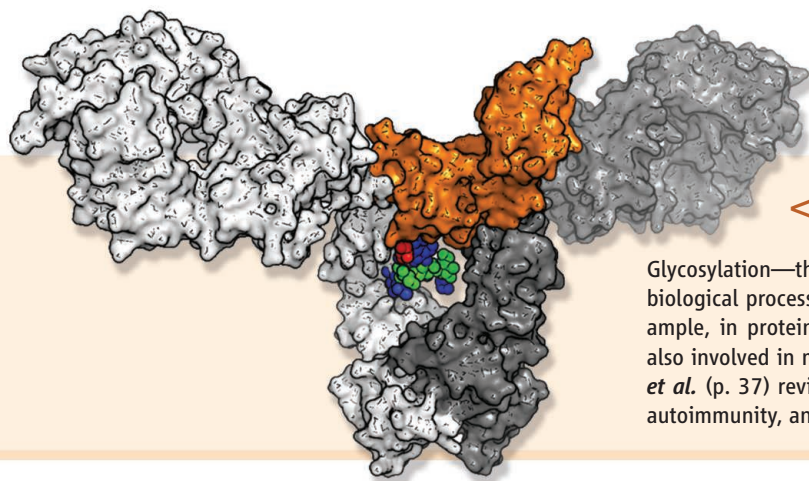
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<< Understanding Glycosylation

Glycosylation—the covalent addition of carbohydrates to proteins—is central to many biological processes. Recent advances in understanding the roles of glycans—for example, in protein folding and immune regulation—have revealed that glycans are also involved in many disease conditions, from cancer to microbial infection. **Dalziel *et al.*** (p. 37) review the current knowledge of glycans in pathogen invasion, cancer, autoimmunity, and congenital diseases.

Bright Lights

Gamma-ray bursts (GRBs), bright flashes of gamma-ray light, are thought to be associated with the collapse of massive stars. GRB 130427A was detected on 27 April 2013, and it had the longest gamma-ray duration and one of the largest isotropic energy releases observed to date (see the Perspective by **Fynbo**). **Ackermann *et al.*** (p. 42, published online 21 November) report data obtained with the Fermi Gamma-Ray Space Telescope, which reveal a high-energy spectral component that cannot be accounted for by the standard external shock synchrotron radiation model. **Vestrand *et al.*** (p. 38, published online 21 November) report the detection of an extremely bright flash of visible light and unexpected similarities between the variations of optical light and the highest-energy gamma rays that indicate a common origin. A detailed analysis of the first pulse of GRB 130427A by **Preece *et al.*** (p. 51, published online 21 November) suggests that existing models cannot explain all the observed spectral and temporal behaviors simultaneously. **Maselli *et al.*** (p. 48, published online 21 November) present x-ray and optical light curves of the burst's prompt emission as well as of its afterglow as recorded by the Swift satellite and a range of ground-based telescopes.

Unadorned Aziridines

Multiple catalytic methods have been developed to make aziridines—strained triangular carbon-nitrogen-carbon rings that function as versatile synthetic intermediates. However, the majority require protection of the nitrogen precursor with a sulfonyl group that is subsequently inconvenient to remove. **Jat *et al.*** (p. 61; see the Perspective by **Türkmen and Aggarwal**) used a hydroxylamine derivative as the nitrogen source together with an established rhodium catalyst to prepare a wide range of unprotected aziridines, with nitrogen bonded simply to hydrogen or a methyl group.

Playing Hide and Seek

Targeted cancer therapies have shown promising results in patients, but few of these drugs provide long-term benefits because tumor cells rapidly develop drug resistance. **Nathanson *et al.*** (p. 72, published online 5 December) show that glioblastoma cells can become resistant to erlotinib, an epidermal growth factor receptor (EGFR)—targeted drug, by eliminating extrachromosomal copies of the mutant *EGFR* gene. After a period of drug withdrawal, the mutant *EGFR* gene reappears on extrachromosomal DNA and the tumor cells become resensitized. The discovery that cancer cells can evade drug therapy by this “hide and seek” mechanism may help to optimize the dosing schedule of erlotinib in glioblastoma patients.

Counteracting Cannabis

What is the role of steroid hormones in vulnerability to addiction? Working with rodents, **Vallée *et al.*** (p. 94) found that all major drugs of abuse (morphine, cocaine, alcohol, nicotine) increase neurosteroid levels, with the active ingredient in cannabis (THC) inducing a particularly large increase. THC and other drugs increased levels of pregnenolone, long thought to be an inactive precursor of downstream active steroids. Pregnenolone antagonized most of the known behavioral and somatic effects of THC.

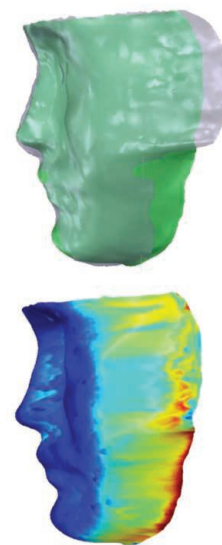
Improving Whole-Genome Screens

Improved methods are needed for the knockout of individual genes in genome-scale functional screens. **Wang *et al.*** (p. 80, published online 12 December) and **Shalem *et al.*** (p. 84, published online 12 December) used the bacterial CRISPR/Cas9 system to power-screen protocols that avoid several of the pitfalls associated with small interfering RNA (siRNA) screens. Genome editing by these methods completely disrupts

target genes, thus avoiding weak signals that can occur when transcript abundance is partially decreased by siRNA. Furthermore, gene targeting by the CRISPR system is more precise and appears to produce substantially fewer off-target effects than existing methods.

Computing an Image

Firing off a burst of laser pulses and detecting the back-reflected photons is a widely used method for constructing three-dimensional (3D) images of a scene. **Kirmani *et al.*** (p. 58, published online 29 November) describe an active imaging method in which pulsed laser light raster scans a scene and a single-photon detector is used to detect the first photon of the back-reflected laser light. Exploiting spatial correlations of photons scattered from different parts of the scene allows computation of a 3D image. Importantly, for biological applications, the technique allows the laser power to be reduced without sacrificing image quality.



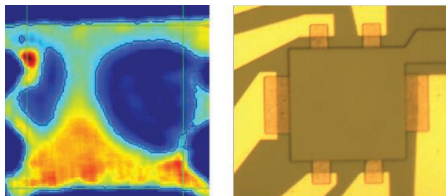
Familiarity Does Not Breed Contempt

Female mating preference is influenced by social familiarity in various species from fish to primates. **Okuyama *et al.*** (p. 91) showed in Japanese rice fish that females prefer to mate with visually familiarized males over unfamiliar males and that this preference is mediated by specific neuromodulatory neurons in the female brain.

Additional summaries

Exciton Liquid

Excitons, bound states of electrons and holes (states “vacated” by electrons), can be found in semiconductors and have long been predicted to form correlated phases at sufficiently large densities and low temperatures. **Stern *et al.*** (p. 55) studied the behavior of spatially indirect excitons, which consist of electrons and holes residing in



spatially separated, but coupled, quantum wells. The excitons were created through a combination of photoexcitation and electric gating. At high enough laser power and low enough temperatures, a new phase with a distinct photoluminescence signature appeared with behavior consistent with that of a classical liquid of excitons.

Guests for Conductors

Thin films of metal-organic framework (MOF) compounds are generally poor conductors because the linking organic groups are usually insulators with little π -orbital conjugation. **Talin *et al.*** (p. 66, published online 5 December) show that infiltrating films of the copper-based MOF HKUST-1 with the conjugated organic molecule 7,7,8,8-tetracyanoquinodimethane created an air-stable material with conductivities as high as 7 siemens per meter.

Why, Oh Y?

The mammalian Y chromosome is a symbol of maleness and encodes genes important for male reproduction. Various deletions of the Y chromosome result in sperm defects and infertility. When haploid male germ cells were injected directly into oocytes, **Yamauchi *et al.*** (p. 69, published online 21 November; see the Perspective by **Capel**) found that living offspring could be derived from male mice whose Y chromosome contribution was limited to only two genes. These two genes are the testis determinant factor *Sry* and the spermatogonial proliferation factor *Eif2s3y*.

Chromosome Condensation

The forces that shape the structure of the highly condensed metaphase chromosomes seen during cell division in eukaryotes are still largely unknown. In vitro evidence suggests that the amino-terminal tails of the histones—such as interaction of the histone H4 tail with H2A-H2B—play an important role in chromosome hypercondensation. **Wilkins *et al.*** (p. 77) used ultraviolet cross-linker amino acids in the histones of bakers’ yeast to show that during early mitosis, phosphorylation of H3 threonine 3 by Haspin kinase recruits the chromosome passenger complex (CPC). The subsequent phosphorylation of H3 serine 10 by CPC allows the recruitment of the deacetylase Hst2p to nucleosomes. Hst2p drives the deacetylation of H4 lysine 16, facilitating the interaction between H4 and H2A-H2B in neighboring nucleosomes, promoting chromatin condensation.

DNA Damage Repair

In human cancers, oncogene activation interferes with DNA replication, leading to DNA replication stress and DNA double-strand breaks (DSBs). **Costantino *et al.*** (p. 88, published online 5 December) identified two subunits of DNA polymerase delta, POL3 and POL4, as critical for survival of DNA replication stress in human cells. Both subunits were required for break-induced replication (BIR), which is required to repair a specific type of DSB, with both subunits possibly required for processive DNA synthesis in BIR. Tandem head-to-tail duplications and fold-back inversions were seen in replication-stressed cells, similar to those seen in human breast and ovarian cancers, suggesting that BIR is important for repairing damaged forks in cancer cells.



Marcia McNutt is Editor-in-Chief of *Science*.

No Windfall for U.S. Science

WITH SEQUESTRATION, 2013 WAS NOT A BANNER YEAR FOR U.S. SCIENCE. THE FEDERAL RESEARCH and development (R&D) budget, at an estimated \$132.8B (billion), was 6.9% below 2012 levels and the lowest it had been since 2002, adjusted for inflation. With October came the government-wide shutdown, when the political parties failed to reach agreement on the 2014 appropriation. After 2 weeks of critical experiments being abandoned, time series suffering gaps, research grants not being funded, and overall loss in the credibility of the U.S. government as a reliable science partner, a temporary budget deal was struck in the form of a continuing resolution that reopened government, but only until January 15 of 2014.

The modicum of good news for 2014 is that a budget deal has been reached by a bipartisan group chaired by Congressman Paul Ryan (R-Wisconsin) and Senator Patty Murray (D-Washington State) that splits the difference exactly down the middle between the House

budget mark of \$967B and Senate mark of \$1.058B. Overall, this agreement restores \$63B to the total federal budget over what would have been expected under the sequestration budget limits. Furthermore, the parties agreed to distribute the \$63B equally between non-defense and defense spending across 2014 and 2015. One might ask why it has taken so many months to craft what appears to be the obvious compromise. But given the recent congressional gridlock that led to sequestration and the shutdown, the fact that there is bipartisan agreement on anything seems nothing short of a miracle. While details still need to be worked out as to how the \$63B will be apportioned to various agencies, it is possible to make some fairly good guesses as to how much will go to R&D using past appropriations history. Absent this deal, requirements under sequestration (primarily on the defense side of the R&D budget) would have likely reduced the 2014 R&D budget to about \$130.1B. With the budget deal, R&D

will likely increase to about \$136B instead, a positive boost of 4.5%.

The 2014 budget will continue what has been a decades-long slide in the ratio of the federal R&D budget to the GDP (gross domestic product). This ratio is often used as a measure of how much a nation values basic research; it has fallen 25% in the last decade alone.

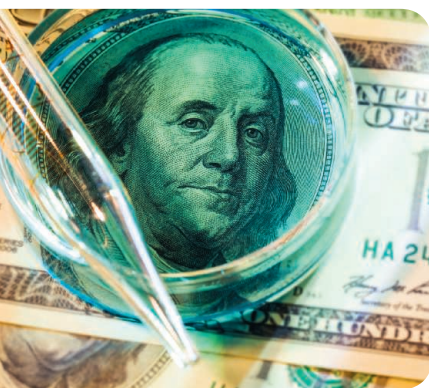
In the meantime, elsewhere internationally, investment in science is rising as nations throughout the world connect investment in R&D to the development of their human capital and to their future prosperity. For example, the European Union's flagship research program, Horizon 2020, is set to receive a nearly 30% boost in 2014. The Chinese government's investment in R&D has been increasing by percentages in the double digits for the last several years and is poised to become the world leader.

One can already see the cascading consequences as federal R&D budgets shrink. The best and the brightest students trained at world-class U.S. universities grow disillusioned and seek other careers or better opportunities overseas for pursuing their research. Research programs are narrowing their scope as budgets decline to maintain reasonable funding success levels, and gaps appear between programs such that some areas of fundamental investigation fall between the cracks. Ultimately, the flow of discoveries from basic research, primarily supported by the federal government, will slow down, as will the pace of innovation.

This erosion of the U.S. scientific enterprise is not in the best interest of the global scientific enterprise, and certainly not in the best interests of the United States. Professional societies can only do so much in terms of advocacy. Congress needs to hear from every U.S. scientist, engineer, technologist, and anyone whose job depends on the innovation pipeline. It is far easier to keep U.S. science world class than to rebuild it.

— Marcia McNutt

10.1126/science.1250035





ENDOCRINOLOGY

It's in Her Eyes

Crustaceans go through pubertal molt to provide the animal with features that are distinct to adults. The androgenic gland hormone (AGH) is important for male differentiation and secondary male characteristics. Females lack AGH, so are considered the default sex for development. Previous work has shown that when the eyestalk is ablated in the females of some crab species, mating and maternal care structures show defects; however, the animals are able to molt and develop into giant immature crabs. In studying the blue crab, *Callinectes sapidus*, Zmora *et al.* now show that the endocrine system and localized activity of a hormone termed crustacean female sex hormone (CFSH) from the eyestalk ganglia are involved in adult-specific development through the control of the pubertal-terminal molt. When CFSH is eliminated, the brooding features, which are important for mating and brooding large clutches, are abnormal. This work shows that the endocrine system functions via a female-specific hormone for the development of adult morphological structures associated with female reproduction, i.e., for mating and brooding. — BAP

Endocrinology 10.1210/en.2013-1603 (2013).

MOLECULAR BIOLOGY

Polymerase Proofreader

For organisms to grow, and their cells divide, the genomes they contain must be replicated quickly and accurately. In eukaryotes, three DNA polymerases handle genome duplication: Pol α , which makes short RNA-DNA primers; Pol δ , which discontinuously replicates the lagging DNA strand and Pol ϵ , which replicates the leading strand, in a largely continuous and highly processive manner, unlike the other polymerases. Hogg *et al.* determine the 2.2 Å resolution crystal structure of the catalytic core of yeast Pol ϵ caught in the act of polymerization—bound to DNA and with an incoming dATP base. As well as the typical fingers, palm, thumb, exonuclease, and N-terminal domains, yeast Pol ϵ has a novel “P” domain that extends from the palm and encircles the DNA, embracing it as it leaves the active site. The P domain facilitates the high intrinsic processivity of Pol ϵ , independently of the processivity clamp protein, PCNA. It also extends the interactions between Pol ϵ and DNA by up to 10 nucleotides. Hogg *et al.* speculate that this much extended interface may contribute to Pol ϵ 's high replication fidelity by allowing Pol ϵ to proofread the newly synthesized DNA for errors up to 45 Å from the active site. — GR

Nat. Struct. Mol. Biol. 10.1038/nsmb.2712 (2013).

SIGNALING

Good Gut Bugs

The trillions of bacteria in the human gut are important for normal digestion and can contribute to disease when their beneficial actions are lost. They also communicate with and modulate the behavior of the intestinal cells that they contact. Jones *et al.* examined the signaling mechanisms by which *Lactobacilli*, bacteria that we consume in cheese and yogurt, stimulate normal proliferation of intestinal cells in fruit flies and mice. These beneficial bacteria caused activation of NADPH oxidase, an enzyme that produces reactive oxygen species (ROS). Oddly enough, production of large amounts of ROS by phagocytes or in the intestine is a defense mechanism for killing bacteria. The smaller amounts produced in response to the *Lactobacilli*, however, had the beneficial effect of promoting cell proliferation needed for a healthy intestine in the flies and mice tested. Patel *et al.* point out in a commentary that the difference between the reaction to pathogenic and friendly bacteria may thus be primarily in the amount, rather than the type, of signal produced. — LBR

EMBO J. 32 10.1038/emboj.2013.224;
10.1038/emboj.2013.244 (2013).

CLIMATE SCIENCE

A Question of Balance

Arctic permafrost is thought to contain twice as much carbon as the atmosphere. As climate warming causes increasing amounts of that permafrost to melt, release of carbon as methane or carbon dioxide (CO₂) may cause additional,

rapid warming. However, it is likely that at least some of the carbon made available by permafrost melting will be used by the vegetation that is appearing in warming regions, but how large a sink that may be is unknown. Lupascu *et al.* report that in High Arctic tundra—an important subset of permafrost terrain, which is experiencing a complex combination of rising temperatures, increasing precipitation, and permafrost degradation—warming alone increases the summertime CO₂ sink strength by up to 55%, but warming combined with wetting increased the CO₂ sink strength by an order of magnitude. Thus, the High Arctic has the potential to remain a strong carbon sink even if the rest of the Arctic permafrost region becomes a net carbon source due to future global warming. — HJS

Nat. Clim. Change 10.1038/
NCLIMATE2058 (2013).

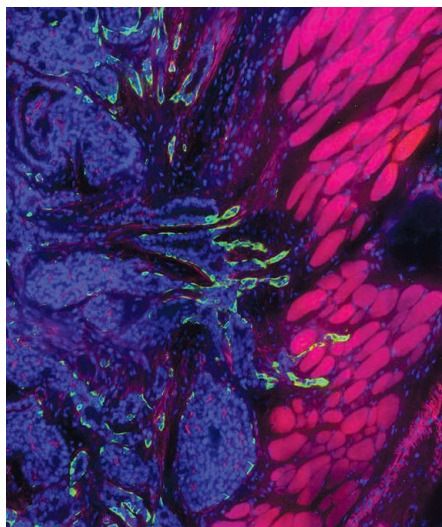


CANCER

Leading the Invasion

Metastasis of solid tumors requires that normally immotile epithelial cancer cells acquire the ability to invade surrounding tissue. Current models depict invasion as a multicellular event dependent on the collective action of stromal cells and specialized, but poorly defined, subpopulations of cancer epithelial cells. Identifying the distinguishing molecular features of the most invasive cells could potentially provide new targets for therapeutic intervention.

Studying breast cancer invasion in vitro, in mouse models, and in human tumor samples, Cheung *et al.* found that the cancer cells “leading” the invasion expressed genes characteristic



of basal epithelium, including *cytokeratin-14* (*K14*) and *p63*. The leader phenotype appeared to be a differentiation state rather than a fixed lineage, as *K14*-negative luminal cells (a mammary epithelial cell type that gives rise to most breast cancers) were found to undergo conversion to invasive behavior in vitro, concomitant with the acquisition of *K14* expression. Notably, shRNA-mediated inhibition of *K14* or *p63* expression blocked the invasive capacity of breast tumors in vitro, suggesting that therapies aimed at disrupting the basal epithelial program might impede metastasis. — PAK

Cell 155, 1639 (2013).

CHEMISTRY

Mirror, Mirror on the Wall

What's the strongest acid of them all? In the case of Brønsted acidity, associated with the tendency to lose a proton, it depends on the surrounding medium. The pK_a scale was originally grounded

in reactivity toward water, but many common solvents are inherently less basic, which allows discrimination of acids that would otherwise all dissociate fully under aqueous conditions. Halogenated carboranes have emerged as the weakest class of conjugate bases in this context; unlike antimony pentafluoride-derived superacid conjugates, they lack a highly Lewis acidic component. Nava *et al.* report the synthesis and isolation of an acid with the fluorinated carborane $\text{CHB}_{11}\text{F}_{11}$ as its conjugate, following up on a report several years ago of its preparation and the vibrational spectrum of an ethyl-substituted analog. Because this compound is sufficiently acidic to cleave alkyl C-H bonds by protonolysis, it was crucial to exclude organic impurities (as well as water) during the steps prior to its ultimate purification by sublimation. Suspension of the solid in hexane was observed to liberate hydrogen at room temperature in 50% yield within 2 hours, a substantial rise in reactivity relative to the previous Brønsted champion, which bore a chlorinated carborane conjugate; butane reacted similarly. — JSY

Angew. Chem. Int. Ed. 10.1002/anie.201308586 (2013).

EDUCATION

Fostering Self-Worth

First-generation college students, where neither parent received a 4-year college degree, tend to perform more poorly and have higher dropout rates than continuing-generation students, who have at least one parent with a 4-year degree. Harackiewicz *et al.* hypothesized that offering first-generation students the chance to remind themselves of their personal values—for example, creativity, career aspirations, or the desire to be independent—may help them to foster their self-worth and thus lead to an improvement in performance and to lower dropout rates. The authors used the values affirmation (VA) intervention, which involves students writing about their most important values, on 798 U.S. students (154 first-generation) in an introductory biology course. Before taking biology tests, students in the VA group were instructed to circle the values most important to them among 12 given values, whereas students in the control group were asked to circle the values least important to them. The VA intervention narrowed the achievement gap between first- and continuing-generation students by 50% and increased retention of first-generation students by 20%. Thus, interventions that change the mindset of students are powerful and can complement interventions that focus on changing the learning environment. — FB

J. Educ. Psychol. 10.1037/a0034679 (2013).

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A Look Ahead for 2014



Gulf Coast, United States

Costs of BP Oil Spill Revealed

Advocates for restoring ecosystems around the Gulf of Mexico are watching for two big breaks that could come in 2014. A federal court is expected to decide how much the oil giant BP will have to pay in Clean Water Act fines for the 2010 *Deepwater Horizon* spill. The total could exceed \$15 billion, and 80% of the funds will be spent on economic and ecological restoration in the five Gulf Coast states. Meanwhile, a separate government-led process will determine how much damage the spill caused to natural resources and how much oil companies owe to repair it. That sum is also expected to run in the billions.

California

Stem Cell Funder Faces Uncertain Future

The California Institute for Regenerative Medicine is pondering its future as it looks for a new director. Created by California voters in 2004 in part to sidestep federal restrictions on embryonic stem cell work, the funding agency will get its last annual installment of about \$300 million from the state this year. It has enough money stockpiled to last a few more years, but to stay in business longer, it could seek funds from another state referendum, as early as this November. Some advocate that the institute, which has lately favored clinical trials over basic research, instead seek money from industrial partnerships.

China

New Hunt for Submerged Relics

China is expected to launch its first dedicated underwater archaeology ship in early 2014 as part of an ambitious push into marine

archaeology. The \$98.8 million, 500-ton vessel will scour for wrecks in coastal Chinese waters and in the South China Sea, according to state news agency Xinhua. Control of that area is disputed by several Asian nations, prompting concern that China is using exploration as a pretext for flexing its naval muscle. Officials counter that the ship is intended to bolster appreciation of the country's cultural heritage by excavating historical relics.

India

Three Years Polio-Free

Barring any surprises, on 13 January India will have gone 3 years without a case of polio, an achievement many never thought possible. With its astounding birthrate, population density, and poverty, India was long considered the most biologically challeng-



ing country in which to eradicate the virus. But after a few weeks of additional surveillance for undetected cases, the country is set to be certified polio-free. Massive vaccination campaigns will still continue; as long as polio is still circulating anywhere, as it is in neighboring Pakistan, the country is at risk of reinfection.

United States

BRAIN Initiative Funding Starts to Flow

The Brain Research through Advancing Innovative Neurotechnologies (BRAIN) Initiative, an ambitious U.S. effort to find new ways to study the brain in action, will start in earnest this year as roughly \$110 million in federal funding begins to flow to researchers. The Defense Advanced Research Projects

Comet Churyumov–Gerasimenko

Rosetta Springs to Action

Europe's Rosetta spacecraft has been speeding across the solar system for nearly a decade in pursuit of comet Churyumov–Gerasimenko. Like the stone that decoded Egyptian hieroglyphics, Rosetta will this year seek to decode the origins of the solar system. Waking on 20 January from 957 days of hibernation, it will close in for an August rendezvous and begin observing the comet, then dispatch a lander called Philae to ride with the nucleus hopefully as long as its close encounter with the sun in mid-2015. Comets are ancient relics of the solar system and may have seeded planets like Earth with water and even the building blocks of life.

Agency will dole out \$70 million in 5-year grants to teams working on devices to cure neurological disorders and restore memory loss. And in December, the National Institutes of Health released a call for grant applications for \$40 million in six "high-priority" fundamental research areas, including novel methods of classifying types of brain cells and determining their role in specific neural circuits.

Mars

Indian Orbiter on Steady Course

India's maiden mission to Mars, the satellite known as Mangalyaan, is healthy and cruising toward a September rendezvous with the Red Planet, says the Indian Space Research Organisation (ISRO). The \$70 million mission, launched on 5 November, will spend its projected 6-month sojourn measuring atmospheric composition and mapping surface features. Among Mangalyaan's instruments is the first dedicated methane sensor for Mars, which will estimate the presence



CREDITS (TOP TO BOTTOM): GERALD HERBERT/AP IMAGES; BIKAS DAS/AP IMAGES; ISRO



or absence of the gas—possibly indicative of carbon-based life—on a planetary scale. But more than anything, says ISRO Chair K. Radhakrishnan, the mission is intended to “demonstrate India’s technological prowess” for future interplanetary missions.

United States

Bring Back the Budget Wars

As much as scientists hated the chronic gridlock on the federal budget, they may soon develop second thoughts about the recently passed 2-year agreement that staves off the budget cuts known as sequestration until 2016. The agreement still gives powerful interests, like the military, the chance to argue for changes to spending provisions they don’t like. And while everybody likes science, no one makes it their top priority.

President Barack Obama’s upcoming 2015 budget request will again propose a healthy increase for research. But his requests haven’t fared well in years past, and there’s little reason to expect a better result this time around.

United States

Will Congress Re-COMPETE?

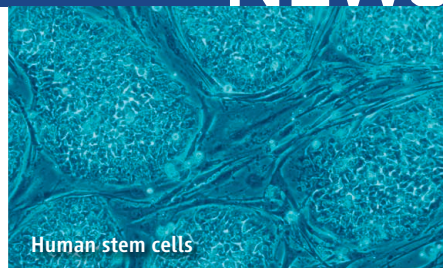
Beyond allocating money, Congress can shape U.S. science policy by creating or killing programs at individual agencies and altering their priorities. This year, Congress is scheduled to take up such a bill, a successor to America COMPETES, that provides

guidance to the National Science Foundation (NSF) and several other research agencies. Science lobbyists expect the Senate to reaffirm federal support for research and science education, but they fear the House of Representatives will push for language that alters peer-review guidelines and hampers NSF’s ability to fund the best research. If that happens, many believe that no bill would be better than a bad bill.

Italy

Fight Over Stem Cell Therapy Endures

The tumult surrounding a controversial Italian stem cell therapy is set to continue. Patients have lobbied hard for the government to fund the therapy, designed by the Stamina Foundation to treat neurodegenerative diseases. In October, the government scrapped a publicly funded clinical trial after a panel concluded that the treatment had no scientific merit. Then last month, in a ruling that shocked many scientists, an Italian court said that the panel wasn’t impartial. This year, the government



Human stem cells

is expected to ask a new expert group to study the treatment’s validity before deciding whether to pull the plug again.

European Union

Overhaul for Clinical Trial Rules

Before May, the European Union is slated to approve a revamp of its unpopular 2001 clinical trials directive. The new regulation will cut down on red tape and require results from every trial to be uploaded to an E.U.-wide database. In a similar vein, the European Medicines Agency has promised to push ahead with its plans to open to the public and scientists its treasure chest of clinical data from marketing authorization applications. But these plans have already been delayed: In particular, two drug companies have sued the agency for divulging data to competitors.



Antarctica

Groundbreaking on the Ice

Two new Antarctic research stations are rising as China and South Korea stake their claims on polar research. A Chinese team reached East Antarctica last month to start building the nation’s fourth Antarctic base, called Taishan. Researchers from many countries are eager to use the summer-only outpost as a launch pad for probing the geological history of the Grove Mountains and the glaciology of the Amery Ice Shelf. Taishan’s main building is expected to be completed next month, and a permanent runway should open in about 2 years. Chinese scientists are also surveying along the western coast of the Ross Sea this season for a place to build their third year-round station.

Meanwhile, the Korean Polar Research Institute plans to open its Jang Bogo research center at Terra Nova Bay in February. The roughly 4000-square-meter facility—South Korea’s second year-round station—is designed to house 16 people in the winter and 60 in the summer. Among the key scientific tasks at Jang Bogo will be monitoring regional climate change.



NATIONAL LABORATORIES

Leading U.S. X-ray Source Goes After Bigger Upgrade

Halfway through the planning process to improve the brightest x-ray synchrotron in the Western Hemisphere, U.S. physicists have decided they want to completely rebuild the facility's particle accelerator.

The new upgrade, more radical than the original plan, would require shutting down for at least a year the Advanced Photon Source (APS) at Argonne National Laboratory in Lemont, Illinois. Some 5600 users in fields ranging from geoscience to structural biology would need to take their work to other machines. The midcourse change also risks restarting the lengthy approval process at the Department of Energy (DOE), which owns the lab and must approve any major renovation.

APS opened in 1996, and its beams helped illuminate the molecular structure of cellular sensors called G-protein-coupled receptors, which won the Nobel Prize in chemistry in 2012. In 2008, Argonne scientists proposed the facility's first major upgrade—incremental improvements to the synchrotron that generates the x-rays and the development of new beamlines to funnel them to users. That plan passed two of the four DOE milestones needed to begin construction. But last summer, a DOE advisory panel urged Argonne to pursue a more extreme makeover.

The rebuild is a better way to ensure that APS stays on top of the heap among x-ray sources, says Ian Robinson, an APS user and nanotechnologist from University College London. "I personally think the first plan was

a mistake from beginning to end," he says. But even Robinson, a fan of the rebuild, says "not all of the 5000 users are going to benefit from the capabilities of the new machine."

The new plan would push APS to the theoretical limit of brightness for a synchrotron by using an approach that only recently has become technologically feasible, says G. Brian Stephenson, Argonne's associate lab director for photon sciences. Scientists in Sweden are applying it for the first time in a lower energy synchrotron called MAX IV, under construction in Lund, which should be the world's brightest when it turns on in 2016.

Synchrotrons accelerate electrons to nearly the speed of light around a circular path. As they bend around the ring, the electrons radiate x-rays, like a wet dishtowel flicking off drops of water when it's twirled overhead. To maximize x-ray production, magnets called undulators shake the beam sideways and cause it to radiate.

The release of radiation spreads the electron beam horizontally. APS designers combat that spreading with beam "optics": The spreading caused by one bending magnet, or dipole, is largely undone by the next one. Still, the electron beam at APS—a 10-micrometer-high and 275-micrometer-wide ribbon—produces a wide, diffuse x-ray beam.

In the new upgrade, each of the ring's 40 pairs of 3-meter-long dipole magnets

Scrap it. Researchers want to completely rebuild the accelerator at Argonne's Advanced Photon Source.

would be replaced with a string of five to eight shorter ones. That change should shrink the width of the electron beam to between five and 19 micrometers, at which point it would have no effect on the x-ray beams. Instead, the width of an x-ray beam would be set by only the wavelength of the x-rays, making APS the "ultimate synchrotron."

The new beams would shine 100 times as intensely as current ones—compared with the sixfold increase originally planned. The beams would also be coherent, with the x-ray photons oscillating in lockstep like those in a laser beam. Coherent beams would be a boon to materials scientists, who could probe materials in novel ways. The improved focus will also benefit nanotechnologists.

But not all APS users need such beams. "We may have to attenuate the beam because it will fry our stuff," says Amy Rosenzweig, a structural biologist at Northwestern University in Evanston, Illinois, who uses APS to analyze protein structures. Structural biologists make up one-third of APS users.

The earlier proposed upgrade was expected to cost \$391 million. The new accelerator is expected to carry a similar price tag. But that's not the only cost. Many of APS's 66 beamlines may not be able to handle the more intense beams. The change would require their owners, many of whom are funded by non-DOE sources such as the National Institutes of Health, to spend millions of dollars on new beam optics and detectors. "Where are they going to get the money?" says Alfonso Mondragón, a structural biologist at Northwestern.

Lab officials hope they won't have to repeat the entire DOE approval process. But some users are less sanguine. "The scope of the project they have now is totally different from the scope of the previous one," says Alan Goldman, a condensed matter physicist at DOE's Ames Laboratory in Iowa and Iowa State University. "Why wouldn't they have to jump through those hoops again?"

Proponents believe that DOE is already on their side. In fact, some think DOE officials asked the advisory committee to study the issue with the hope its report would kick-start the idea. Users would like DOE and Argonne to reach an agreement by this spring and say there's no time to waste: Scientists at the rival European Synchrotron Radiation Facility in Grenoble, France, are mulling a similar rebuild. **—ADRIAN CHO**

SCIENCE POLICY

U.S. Tax Credit: Boondoggle or Boon for Research?

For the sixth time in 21 years, Congress has allowed a popular tax break that encourages U.S. companies to invest in research to expire.

The 31 December lapse of the research and experimentation (R&E) tax credit, estimated to be worth \$7 billion to U.S. firms last year, marks the latest twist in the credit's tangled 32-year history. There's little doubt that Congress will eventually renew it in some form. But the lapse has reignited debate about whether the measure is a costly corporate windfall or a vital incentive for research—and whether it should be made permanent.

Supporters are fuming over the credit's latest demise. "I have heard it said that in Washington it isn't one damn thing after another, it's the same damn thing over and over—and the repeated expiration of the credit is a prime example," says economist Jerry Jasinowski, a former president of the National Association of Manufacturers, who believes that Congress should make the tax break permanent. Lawmakers' "blasé treatment" of the credit, he says, "is harming U.S. competitiveness by creating uncertainty for businesses trying to plan investments."

Critics of the credit, however, are dubious. The subsidy "is riddled with problems," argued Citizens for Tax Justice (CTJ), a Washington, D.C.-based advocacy group, in a report issued last month. The group favors redirecting "the billions of dollars that it costs into ... truly scientific research, which businesses rarely engage in because the payoffs often take years to arrive." At a minimum, it says, the credit needs to be "reformed dramatically."

Such dueling is likely to intensify this year. Lawmakers are expected to try to eliminate many corporate tax breaks in the first major rewrite of U.S. tax law since 1986. But there appears to be support for retaining some kind of R&E credit, which has been renewed temporarily 15 times. (It lapsed for a full year from 1995 to '96.) Lawmakers from both parties and the White House have already floated various proposals to revise or expand the credit.

Few quibble with the economic theory used to justify the tax break. Economists argue that a private company is rarely able to capture all of the benefits created by its investment in research; some "spill over" into society at large (for instance, in the form of better trained scientists who may move to other firms). So companies tend to spend less on research than is optimal for society.

The credit is supposed to increase spillover by rewarding R&E companies for spending more on research than they might otherwise. For example, the U.S. version allows a company to write off up to 20% of what it spends on research above a "base amount" calculated using the firm's spending history. A hypothetical firm that spent \$100 million on research in 2013 could get about

Several lawmakers have called for making it easier for small businesses to claim the credit by streamlining paperwork. In a 2012 report for the Center for American Progress, a left-leaning advocacy group, economists Laura Tyson and Greg Linden called for simplifying and broadening the definition of covered research, in part to avoid expensive and time-consuming disputes between companies and government tax collectors.

Such changes, advocates say, could help prevent the United States from falling even further behind other nations that have been sweetening their research tax incentives. In 2012, U.S. incentives ranked 27th on a list of 42 national policies rated by the Information Technology and Innovation Foundation, down four places from 2007. (India, France, Portugal, and Spain topped the list.)

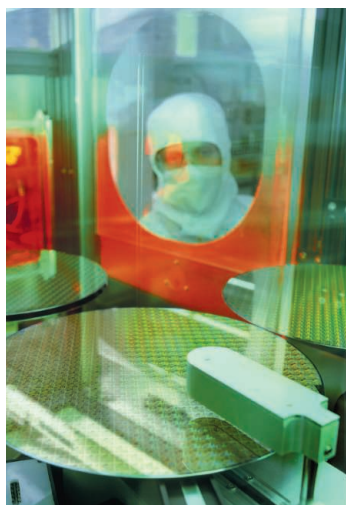
The credit's skeptics, however, say that it often rewards companies for doing research they would have conducted anyway. Rather than broadening the research definition, Congress should clarify and tighten it, says CTJ's Steve Wamhoff. "If Americans are going to subsidize all this, then it should be for research that actually has possible social benefits."

Critics also want to do away with a company's ability to claim the credit years after it has finished the research.

That's a strategy heavily promoted by some accounting firms, Wamhoff notes. "You can't argue [that] the credit is this big incentive for additional research if the company didn't know it was eligible," he says. The nation might be better off, Wamhoff says, if Congress killed the credit and used the money for targeted, direct research grants to companies and universities.

That's an unlikely scenario, although the tax-writing committees in the House of Representatives and the Senate haven't yet spelled out their plans for the credit. And making it permanent could be difficult; analysts estimate that could cost \$100 billion over the first 10 years, a significant amount at a time lawmakers are looking for ways to shrink the deficit.

—DAVID MALAKOFF



Give Them Credit

U.S. companies claiming research tax credit for 2008–2011 (in millions).

General Motors	\$ 774
Boeing	\$ 650
Intel	\$ 544
Google	\$ 332
Apple	\$ 295
Bristol-Myers Squibb	\$ 295
Texas Instruments	\$ 215
Cummins	\$ 171
Lockheed Martin	\$ 157
Hewlett-Packard	\$ 154

\$8 million taken off its tax bill, according to the Congressional Research Service. Studies suggest that every dollar in R&E tax relief can prompt a business to spend at least an additional dollar on research, although critics question such claims.

U.S. firms like the credit: They filed nearly 13,000 claims totaling \$8.5 billion in 2010, the CTJ report estimates. (Not all were allowed.) Big companies appear to benefit most; firms with annual gross revenue greater than \$250 million accounted for 82% of the 2010 claims.

Advocates of the credit, including high-tech companies and drug firms, want it made permanent and tweaked. Many support an Obama administration proposal to increase one form of the write-off to 17% from 14%.

SCIENTIFIC PUBLISHING

Google Scholar Wins Raves—But Can It Be Trusted?

Over the past year, Jonathan Eisen's reading habits have changed dramatically. For most of the past 2 decades, he has kept up with scientific literature primarily by combing PubMed, the vast trove of online biology abstracts. But these days Eisen, an evolutionary biologist at the University of California, Davis, discovers research relevant to his own work without even looking for it.

The insightful librarian helping keep Eisen up to speed is Google Scholar, a free academic search service maintained by the California-based company. Google Scholar has been studying Eisen closely. It keeps track not only of his own 300 papers and the key words within them—Archaea, *Plasmodium*, phylogenomics—but also the 38,000 citations to his work in published papers, preprint abstracts, books, and even conference posters. Like a scientific version of the Netflix movie recommendation engine, Google Scholar scours the Internet, scoring all scientific documents for their predicted interest to Eisen, and then sends him a weekly e-mail of recommended reading.

Eisen is part of growing crowd of converts. "Google Scholar is having a great impact on the research-seeking behavior of researchers," says Nicolás Robinson-García, a bibliometric researcher at the University of Granada in Spain. Robinson-García claims that Google Scholar's compendium of articles is at least as comprehensive as the leading commercial academic search databases—Thomson Reuters' Web of Science and Elsevier's Scopus—and for many disciplines in the social sciences and humanities, even better. And by all accounts, it is gobbling up market share. "Google is the dominant referral source for online journal articles, far surpassing the amount of traffic driven by other discovery tools," says David Crotty, senior editor at Oxford University Press in New York City.

But researchers aren't just using Google Scholar as a search engine. Its algorithm provides citation metrics that quantify the impact of their own published work, and these numbers are becoming part of a standard scientific CV. This byproduct of

Google Scholar has sparked a new concern: Because it includes sources from across the Internet—not only vetted journals—and has no human curators, Google Scholar's citation metric can be easily gamed.

Robinson-García is part of a team that demonstrated that vulnerability by placing six fake papers with long lists of citations to their own work on a webpage within the University of Granada Internet domain. Google Scholar's algorithm dutifully tallied them as real citations and in a matter of weeks, their Google Scholar citation scores rose significantly. The team's findings appeared

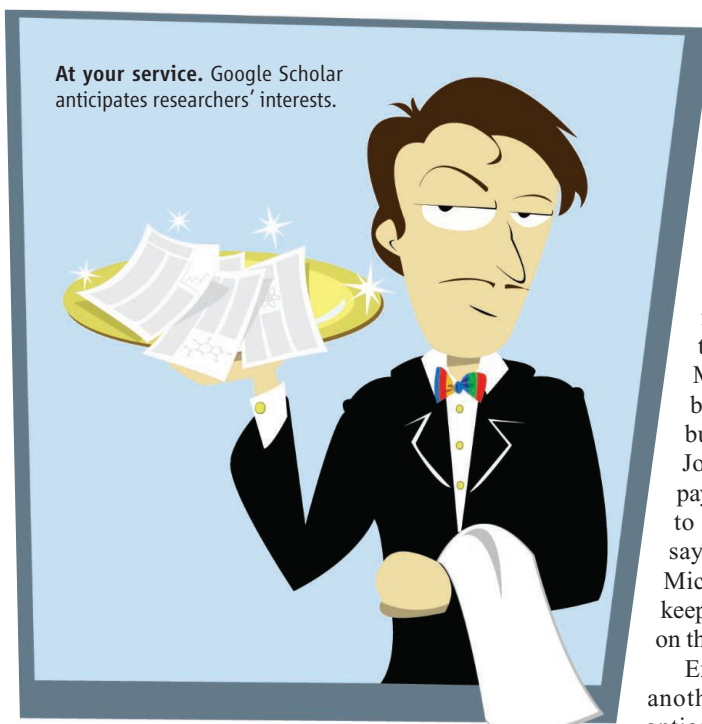
its development, labels efforts to skew the citation metric as "spam." So far, he says, "the level of spam in scholarly articles remains low." Acharya chalks this up to the "large penalty that would come with being caught" rigging the system in scholarly communities. But if social norms fail to keep academic cheaters in check, he says, "we can and will adjust the level of spam-handling." However, he says, Google has no intention of revealing its algorithm, in part because it is tied up with the company's core search engine.

Google Scholar's ascendance may soon be challenged. "Microsoft is still working in this area too," says Anne-Wil Harzing, the creator of Publish or Perish, a citation analysis tool that uses data from Google Scholar. A service called Microsoft Academic Search is expanding rapidly to cover all academic domains. Meanwhile, Thomson Reuters and Elsevier have a strong financial incentive to keep their services competitive. Many universities are bound by nondisclosure agreements, but Cornell University librarian John Saylor says his institution pays \$155,000 per year for access to Web of Science. As Harzing says, "having both Google and Microsoft in this field will surely keep Thomson Reuters and Elsevier on their toes!"

Experts say the competition has another benefit: giving scientists options, so that they don't come to depend on just one service, such as Google Scholar, that may be vulnerable to corporate downsizing. There are "persistent rumors that Google is de-emphasizing or even dismantling the Scholar team," says John Sack, founding director of HighWire, an online publishing platform for more than 1300 journals, including *Science*.

Although Google Scholar generates no direct income, Acharya is upbeat about its future. While he declines to reveal usage figures, he claims that the number of users is growing worldwide, particularly in China. And the Google Scholar team is expanding, not contracting, he says. "Rumors of our demise are greatly exaggerated."

—JOHN BOHANNON



online on 11 November in the *Journal of the American Society for Information Science and Technology*.

If Google Scholar were to provide a breakdown of the sources it tapped for its citation metric, Robinson-García says, "our fraud could have been easily detected." That lack of transparency is a deal breaker for some bibliometric researchers, including Rodrigo Costas Comesaña of Leiden University in the Netherlands; he calls Google Scholar "an unmanageable tool" for citation analysis.

Google counters that critics are overstating the problem. Anurag Acharya, a co-founder of Google Scholar who leads

ENVIRONMENTAL POLICY

EPA Science Report Signals Start Of Wetlands Battle

Intermittent streams, swamps that vanish by midsummer, isolated ponds—it's easy to overlook these small, short-lived water bodies. But a recent report by Environmental Protection Agency (EPA) scientists makes the case that they deserve the same protections against pollution and development that the Clean Water Act offers to larger rivers and lakes.

The report, which received preliminary approval from an EPA Science Advisory Board (SAB) panel on 18 December, is the latest salvo in a decadelong battle—waged in the courts and in Congress—over ephemeral water bodies, which have a multitude of ecological functions. In a landmark ruling in 2006, the Supreme Court had concluded that the Clean Water Act and allied environmental regulations don't apply—unless the government can show that a water body has a meaningful physical, chemical, or biological connection to larger “navigable” waterways.

The new report synthesizes more than 1000 publications to establish those links. It is “a remarkable technical achievement,” says SAB member Judson Harvey, a hydrologist with the U.S. Geological Survey. And it provides key scientific backing for a new stream and wetland protection rule that the Obama administration is preparing.

The new rule will be controversial. Although it is still a draft, and hasn't been formally released, it was leaked to Bloomberg

BNA in September and is already drawing fire. Landowners and industry groups are lining up to block it. Representative Lamar Smith (R-TX), chair of the House of Representatives science committee, has called it “a massive power grab of private property” that hasn't gotten proper scientific scrutiny.

The conflict is rooted in the Clean Water Act of 1972, which requires anyone wanting to dredge or fill a stream or wetland to get a permit from the federal government. (Many agricultural activities are exempt.) For decades, U.S. officials and the courts held that the law applied not just to the “navigable waters” mentioned in the act, but also to all the smaller streams, wetlands, and ponds connected to them in a variety of ways.

Numerous groups challenged that interpretation, however, and in the 2000s the U.S. Supreme Court dealt the government several setbacks. In a 2006 split decision, for instance, it ruled that EPA could not regulate any waters unless it demonstrated that they had a “significant nexus” with downstream navigable waters, such as affecting their physical, chemical, or biological integrity.

Since then, confusion has reigned over the legal status of ephemeral streams and isolated wetlands. In 2010, EPA began to try to clarify matters by writing the new rule. It calls for the Clean Water Act to cover all tributaries, headwater streams, and “adjacent” wetlands.

Other waters without an obvious hydrologic connection to the tributary system could be covered on a case-by-case basis.

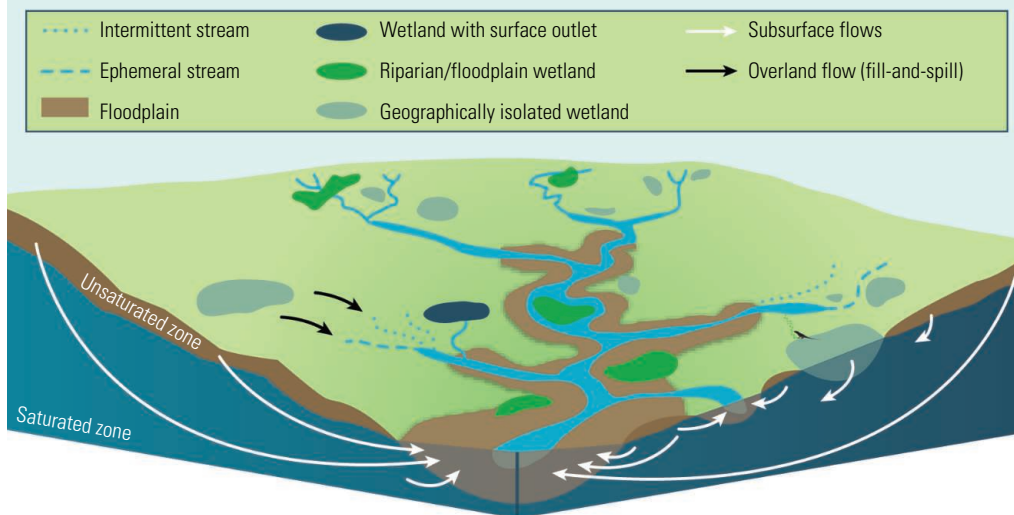
When EPA sent the draft rule to the White House's Office of Management and Budget for review on 17 September, the agency also released for public comment its synthesis of the peer-reviewed science that underpins the rule. It concludes that all tributaries, even if they flow intermittently, pass the Supreme Court's test. They purify water headed to downstream waters and provide habitat for organisms that move back and forth, for example. So do wetlands and ponds located in river floodplains. In addition, the report stresses the importance of considering small streams or wetlands as a group; although each might not have a large impact by itself, together they can have a significant impact on downstream waters.

SAB agreed that many types of wetlands should “categorically” fall under EPA's jurisdiction. EPA scientists hedged, however, on the question of whether wetlands or open waters that sit outside of river corridors are clearly connected to regulated waterways. “We do think there are common processes” that would provide a nexus, said Laurie Alexander, an EPA ecologist who helped write the document, at the SAB meeting. Those processes include removing pollutants, supplying nutrients, and providing habitat for juvenile animals. But government regulators would still need to decide case by case whether these water bodies, such as playa lakes in the dry American Southwest, are covered by the new rule. Most members of SAB urged EPA to reconsider this conclusion, arguing that there was sufficient evidence to categorically include certain types of these wetlands.

Some observers say that EPA's science report falls short. It “fails to deal with the hard issue of finding the edge of connectivity,” or just where any regulatory authority should end, says Deidre Duncan of the Waters Advocacy Coalition, which includes about 30 trade associations opposed to the rule. It does not, for instance, address how large a flood should be used to define a river's floodplain, she noted.

The science is hardly likely to quell criticism of the new rule. Once it is released, Jan Goldman-Carter of the National Wildlife Federation's advocacy center in Washington, D.C., predicts “a historic fight.” —ERIK STOKSTAD

Wet Connections



Go with the flow. Underpinning a politically contentious regulation, EPA scientists lay out the physical, chemical, and biological connections between many kinds of water bodies.

DRUG INDUSTRY

For Mexican Antivenom Maker, U.S. Market Is a Snake Pit

When Jim Harrison was bitten on his hand last summer as he was preparing to milk a western diamondback rattlesnake for its venom, he knew what would happen if the wound went untreated. In a matter of hours, his arm would swell to several times its normal size. Blood vessels would burst, turning his skin black and blue. The excruciating pain would last for days. He could lose the arm and, if the hemorrhaging spread to his brain, suffer a stroke.

But Harrison, director of the Kentucky Reptile Zoo in Slade, had a remedy that is unavailable to most Americans. He grabbed some vials of Anavip, a pit viper antivenom made in Mexico by a firm called Instituto Bioclon, and rushed to the nearest hospital. (The pit viper family includes rattlesnakes, copperheads, and cottonmouths.) Anavip can't be sold legally in the United States; Harrison had access to it because Kentucky Reptile Zoo has a license to stock exotic antivenoms from around the world that have not been approved by the U.S. Food and Drug Administration (FDA).

Bioclon, and many snakebite experts, would like to change the status quo. But the company's January 2012 application for FDA approval to sell Anavip in the United States has led to a nasty cross-border battle. In an 11th-hour bid to block that move, BTG, the maker of CroFab, the only pit viper antivenom legally sold in the United States, filed a complaint in October with the U.S. International Trade Commission asserting that Anavip and its veterinary equivalent, ViperSTAT—made by another Mexican company, Veteria Labs—infringe its patent on antivenom compositions. BTG, based in London, has asked the trade commission to permanently ban Anavip and ViperSTAT from being imported to the United States. The trade commission launched an investigation on 29 November and later this month will set a date for its ruling.

Harrison's case suggests that BTG has good reason to see a threat to its pit viper antivenom monopoly. The herpetologist chose Anavip over CroFab because he assumed the Mexican product would have

a more sustained effect, freeing him from return trips to the hospital for repeat doses of CroFab. Indeed, Harrison quickly made a full recovery. (Kentucky Reptile Zoo has sold venom to BTG and also works with Bioclon's U.S. distributor.)

Antivenom researchers contend that other snakebite victims should have the same choice Harrison did. "I want my patients to have the biggest variety of options available at the lowest cost," says Leslie Boyer, a physician and antivenom researcher at the University of Arizona in Tucson who has led company-funded clinical trials of both Anavip and CroFab. She worries that the trade commission will rule against Bioclon—or "that this is going to be such an expensive process" that Bioclon "will give up."

All antivenom recipes start out by injecting venom into a mammal. (BTG

severe allergic reactions known as serum sickness. For half a century, the standard treatment for pit viper bites in the United States was a whole-IgG antivenom. When Boyer administered it to snakebite victims in the 1980s and 1990s, about 40% experienced "brutal" aches and pains, she says. Many were readmitted to the hospital with serum sickness about a week after being treated.

When FDA approved CroFab in 2000, "it was like a godsend," says Thomas Arnold, medical director of the Louisiana Poison Center in Shreveport. To make CroFab, BTG uses the enzyme papain to sever the IgG molecule above its middle joint, cutting loose the serum sickness-inducing Fc (which is discarded), while separating the arms of the Y into free-floating single Fab fragments. Bioclon employs a similar strategy, using the enzyme pepsin instead of papain. But pepsin makes the cut a bit lower on the Y, leaving the antigen-binding arms connected in a larger F(ab')₂ fragment (see diagram, next page).

In documents submitted to the trade commission, Bioclon and its distributor Laboratorios Silanes assert that leaving the arms connected gives Anavip a competitive edge. CroFab's smaller, single Fab fragments are quickly filtered out of a patient's blood, sometimes before they bind and neutralize all the venom toxins, explains Lourival Possani, a molecular biologist at the National Autonomous University of Mexico's Institute of Biotechnology in Cuernavaca, who has worked with Bioclon.

In a Bioclon-funded study published in the November issue of *Toxicon*, Boyer and colleagues reported that pit viper venom levels rebounded to as high as 25% of their pretreatment levels in the blood of four of six patients treated with CroFab; two required extra doses of the drug. Venom levels did not rebound in any of six patients treated with Anavip, and none needed additional doses.

BTG's patent, however, lays claim not just to the single Fab fragments in CroFab but also to methods for producing connected F(ab')₂ and whole-IgG antivenoms from horses. "We believe [Anavip] absolutely infringes our



Handle with care. When herpetologist Jim Harrison was bitten by a western diamondback rattlesnake, he opted for a Mexican-made antivenom not available for sale in the United States.

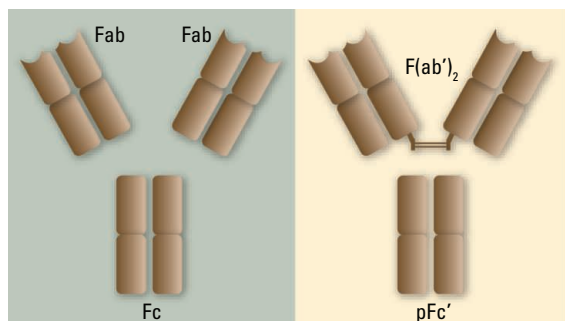
uses sheep; Bioclon opts for horses.) As doses ramp up over 6 to 9 months, antibodies against the venom develop and are harvested regularly from its blood. These immunoglobulin G (IgG) molecules are shaped like a capital Y; the two arms, called Fab fragments, bind to toxins in the venom, neutralizing it. Two of the four antivenoms approved in the United States—to treat coral snake and black widow spider bites—are composed of whole IgG molecules.

Both CroFab and Anavip try to solve a drawback of the whole-IgG approach: The stalk of the Y, called an Fc fragment, is packed with animal proteins that can trigger

patent” and has “piggybacked off our ideas,” says Andy Burrows, BTG’s director of investor relations. In their submission to the trade commission, Bioclon and Laboratorios Silanes stress the difference between Fab and $F(ab')_2$ antivenoms and state that they haven’t yet sold Anavip on the open market in the United States.

Access to a small but lucrative market hinges on the outcome of the trade commission’s investigation. In the United States, about 5500 people are bitten each year by a pit viper of some kind, according to Burrows. Depending on a victim’s body size and the amount of venom injected, a typical bite requires 20 to 25 vials of CroFab, which BTG says it sells to hospitals and distributors at \$2000 per vial. After hospital markups on CroFab and intensive care charges, Boyer says, “it’s very common for the actual bill that a person receives to be greater than \$100,000.”

In Mexico, Anavip and Bioclon’s antivenoms against coral snakes, scorpions, and black widow spiders sell for less than



Fab prize fight. In the left corner, a pit viper antivenom with free-floating Fab fragments. The challenger’s antigen-binding arms remain connected (right).

\$100 a vial. Possani says the price reflects the lower costs of drug approval in Mexico and higher demand. Scorpion stings and snakebites are serious public health issues in Mexico, and the country is the world’s largest antivenom producer, Possani says.

In 2011, Bioclon’s scorpion antivenom, Anascorp, became the first Mexican drug to win FDA approval. The experience was demoralizing, says a Mexican scientist who works with Bioclon and requested anonymity. Bioclon funded 12 years of clinical trials before applying, forcing its U.S. distributor,

Rare Disease Therapeutics Inc., to price Anascorp so high—\$3500 per vial—that it is worth the cost only for the most severe, potentially life-threatening scorpion stings, according to a recent study in *Toxicon*. And that high price comes even after Bioclon passed off a portion of the costs of pursuing FDA approval to consumers back home; Anascorp’s price quintupled in Mexico since the clinical trials began, says the Mexican scientist. “It’s just tragic” that Mexican scorpion sting patients, who are often poor and live in rural areas, “are subsidizing the U.S. market,” Boyer says.

The Mexican scientist assails the FDA approval process as “too expensive, in both money and effort.” He hopes that BTG’s complaint will inspire Bioclon to abandon the U.S. market and focus on developing antivenoms for Central and South America, the Middle East, and especially Africa, where Bioclon is already working with the World Health Organization. “My job is to continue making antivenoms,” he says. “If they can’t be used in the U.S., fine. That’s not my problem.” Snakebite victims this side of the border might not agree.

—LIZZIE WADE

AGRICULTURE

Global Research Network Raises \$1 Billion for Its Centers

Internal reforms and rising public interest in food security have helped an international network of agricultural research centers achieve an ambitious goal of doubling its budget in 5 years, boosting efforts to help farmers and improve crops.

After playing a central role in the Green Revolution, the Consultative Group on International Agricultural Research (CGIAR) system fell into decline during the 1980s and 1990s. Like many national agricultural research centers, the budget for CGIAR’s 15 centers flattened as infrastructure deteriorated and top scientists left. “We lost ground in the work to provide farmers with the tools they need,” says Sara Boettiger of the University of California, Berkeley, who retired this past April as chair of CGIAR’s International Maize and Wheat Improvement Center in Mexico.

But recent years have seen a remarkable turnaround, and on 17 December CGIAR announced that it had reached its \$1 billion target. The money came from outside sources, mainly development agencies in industrialized countries and private philanthropies. “It’s great news,” says economist Prabhu Pingali of Cornell University, who led the agricultural

development program at the Bill & Melinda Gates Foundation until May 2013. The increased funds have already allowed the 15 centers to scale up work aimed at helping small farmers.

CGIAR’s recovery was aided by the food price crisis in 2008 and by a 2006 initiative in agricultural development by the Gates Foundation. In 2010, CGIAR streamlined its management structure to better coordinate the research strategies of its centers. “That resonated with donors,” says Jonathan Wadsworth, executive secretary of the CGIAR Fund, which was created to disperse funds to centers. “We’ve been able to show them the research plans, show them the priorities, what [the centers] aim to achieve with added resources.”

The increased funding has also allowed centers to focus on coping with climate change and improving nutrition and health. One new project involves breeding a sweet potato enriched with a vitamin A precursor. Another effort uses satellite imagery to help make crop insurance programs more effective. New policy research is looking at whether raising the price of electricity for

irrigation in the Punjab will save energy and water. CGIAR scientists are also now working to boost the photosynthetic efficiency of rice by changing metabolic pathways in the plant.

The centers must work around spending restrictions imposed by donors. And there’s little money available for new infrastructure or initiatives such as upgrading information technology systems to provide greater public access to the centers’ databases.

To address those problems, a few centers have been doing their own fundraising. In February, the maize and wheat institute cut the ribbon for 5500 square meters of research labs and greenhouses built with \$25 million from the Gates Foundation and the Carlos Slim Foundation.

Wadsworth says an upcoming independent review will look at whether the group’s budget needs to grow further. Some say any additional money for agricultural research should go to national centers, which often partner with CGIAR centers to adapt crops to local conditions. “The missing gap here,” Pingali says, “is the capacity of the national systems to absorb the knowledge coming out of the [CGIAR].”

—ERIK STOKSTAD



Monumental Roots

The great stone monuments of prehistoric Britain, including Stonehenge, were born in a wave of innovation that apparently began on a remote Scottish island

MAINLAND ISLAND, ORKNEY ISLANDS, U.K.

—To reach this hilly, windswept island in Scotland's far north, you must either catch a flight on a prop plane or else drive for hours through the fog-shrouded Scottish Highlands and cross 40 kilometers of often-choppy seas by ferry.

Once here on the largest of Orkney's 70 islands, you'll find peace and quiet. The mostly treeless hills are dotted with grazing cattle and scattered houses. But 5000 years ago, this faraway landscape was a center of Britain's new stone age—what we now call the Neolithic. Thousands of people gathered in the shadow of one of the world's most spectacular clusters of stone monuments, according to recent excavations and dating.

Sometime around 3200 B.C.E., in the middle of a narrow isthmus that divides a freshwater loch from a salty one, hundreds of people labored to construct a complex of elaborate stone buildings covering at least 10,000 square meters, more than two American football fields. Called the Ness of Brodgar, its thick stone walls, richly adorned with mysterious engravings, may have served as a ritual center not only for Orkney islanders, but also for pilgrims from the Scottish mainland. A few hundred years later, on the south end of the isthmus, the Orkney people erected the Stones of Stenness, a dozen stone pillars up to 6 meters tall, arranged in a circle. A much larger monument, the 104-meter-diameter Ring of Brodgar, rose soon after at the isthmus's

northern end. Teams of laborers dragged 60 monoliths, up to 4.5 meters tall, from quarries as far as 15 kilometers away.

A flurry of discoveries and research across the British Isles is giving archaeologists a new perspective on just why ancient people erected these statements in stone. At Stonehenge, for example, important new insights are coming not just from the stone pillars themselves, but also from a once-bustling settlement 3 kilometers away (see sidebar, p. 20). The work emphasizes how monuments served as social glue to bring geographically dispersed communities together in ritual activities. The process of megalith construction may have been as important as the grand final product. "It may be the act of building that's important, bringing people

CREDIT: DOUG HOUGHTON/ALAMY

Stone sentinels. Orkney's Ring of Brodgar has been standing for nearly 5000 years.

together and sharing and pooling labor," says Mark Edmonds, an archaeologist at the University of York in the United Kingdom.

The new findings also tell a surprising story about the origins of Britain's megalithic monuments ("mega," meaning huge, and "lithic," or stone): They started here on Mainland Island in Orkney, along with new styles of architecture and a special kind of pottery. From this remote, 520-square-kilometer island, these innovations swept nearly every corner of Britain and Ireland, culminating in the famous monuments of Stonehenge and Avebury in southern England. "We're looking at a fairly major transformation across Britain, the impact of a whole way of life, religious and social, which comes out of Orkney," says archaeologist Michael Parker Pearson of University College London (UCL), who has led recent excavations at Stonehenge and surrounding monuments. "Orkney was a place of synthesis, where Neolithic worlds came together."

Farming arrives, dirt mounds arise

People began erecting stone monuments as early as 11,000 years ago, as vividly attested by monoliths carved with strange animal images at the site of Göbekli Tepe in southeastern Turkey (*Science*, 18 January 2008, p. 278). Göbekli Tepe was erected on the very cusp of the so-called Neolithic Revolution, when farming began to spread. But nearly all other stone monuments are later, from the Neolithic period, and most archaeologists think the advent of farming somehow made megaliths either possible or desirable.

Back in the late 1990s, Richard Bradley, an archaeologist at the University of Reading in the United Kingdom, argued that most hunter-gatherers see themselves as one with nature, not apart from it. So they wouldn't think of altering the landscape by erecting stone structures, whereas farmers are all about changing nature. The contrasting worldviews "make very good sense," Bradley says. And there's no doubt that building in stone took off as farming spread to Europe beginning about 8500 years ago. That's when people began burying their dead in stone chambers and erecting stone pillars, especially along Europe's coasts. For example, early farmers

aligned thousands of menhirs (standing stones) at Carnac in Brittany.

But truly massive monuments often lagged behind the beginnings of farming, as a new dating program led by archaeologist Alasdair Whittle of Cardiff University and dating expert Alex Bayliss of English Heritage's London Office has shown. Stone monuments are notoriously hard to date, because radiocarbon techniques require organic material. So archaeologists look for datable remains among associated animal bones, charcoal, or artifacts.

Bayliss and others also apply powerful mathematical techniques called Bayesian statistics to both newly gathered and older dates, boosting accuracy (*Science*, 15 September 2006, p. 1560). When applied to Britain's earliest monuments,

this approach can pinpoint construction to within 40 years or less, allowing Whittle and Bayliss to track monument building generation by generation. The approach "is superb, agenda setting, and groundbreaking," says archaeologist Andrew Jones of the University of Southampton in the United Kingdom.

The team found that farming practices such as cereal cultivation and animal domestication, and the appearance of pottery and timber houses, all began in southeast England a generation or two before 4000 B.C.E., then spread across Britain. But at first these farmers built only smaller structures, like tombs, in stone. "You seem to get a gap before the first appearance of monumentality," Whittle says. He and others think that as farmers migrated into the British Isles from the European continent, perhaps recruiting resident hunter-gatherers to the new way of life, it took several generations for farming populations and social complexity to develop to the point that giant monuments were both possible and meaningful.

In Britain, the first great monuments weren't made of stone but of earth.

Online

sciencemag.org

Podcast with author Michael Balter and slideshow of monuments (http://scim.ag/med_6166).



Mysterious meanings. The interior walls of the Ness of Brodgar were decorated with "butterfly"-shaped symbols.



Life and Death at Stonehenge

DURRINGTON WALLS, U.K.—From all over Britain they came by the thousands, with their families, their pigs, and their cattle, to this huge complex of earthen and wooden monuments by the River Avon, known today as Durrington Walls. Inside a circular earthen bank and ditch, 500 meters in diameter, stood a smaller circle of dozens of stout, upstanding timbers. In the center, the body of a venerated chief lay in state. The pilgrims feasted to his triumphs and to his memory, roasted their cattle and their pigs, and then the procession began.

Thousands marched down the short avenue to the river. The chief's body was loaded into a waiting boat, and a smaller contingent pushed off down this tortuous stretch of the Avon. A few hours later, the burial party alighted on the riverbank, joined by new throngs. Together they marched down another, longer avenue to the somber stone megalith now called Stonehenge. There, the body of the chief was placed atop a flaming pyre, and his spirit joined the ancestors.

This scenario is imaginary, but it's also completely consistent with new studies of the monuments and the animal teeth and bones buried among them. The findings are finally bringing Stonehenge, the most dramatic expression of the megalith movement that swept the British Isles 5000 years ago (see main story), out of the realm of mystery, and they are confirming new ideas about its ritual purpose. "It's nice to think that what started out as a theory turned into

fact," says archaeologist Michael Parker Pearson of University College London (UCL), who has led digs around Stonehenge.

The new data support Parker Pearson's picture of Stonehenge as the place of the dead, and Durrington Walls as the place of the living. At Stonehenge, archaeologists have found more than 60 cremation burials, for example, but few animal bones or residences. At Durrington Walls, they have recovered more than 80,000 pig and cattle bones, but only three fragments of human remains. Stonehenge and Durrington Walls "were exactly the opposite," Parker Pearson says.

The two monuments, 3 kilometers apart as the crow flies, were built about the same time, 4600 years ago, according to dates on a pig bone and antler pick, first reported in 2008 (*Science*, 27 June 2008, p. 1704). Researchers also discovered a short earthen roadway from Durrington Walls to the Avon, resembling Stonehenge's longer avenue to the river and showing that both monuments were connected to the river and so to each other. The life versus death model "holds up very well," says Joshua Pollard, an archaeologist at the University of Southampton in the United Kingdom.

The rituals at the monuments were sometimes accompanied by great feasts, possibly around the winter solstices. (Stonehenge is aligned to the winter and

summer solstices.) Zooarchaeologists can estimate when a pig was killed by the amount of wear on its teeth, and unpublished results show that most were killed in winter, says zooarchaeologist Umberto Albarella of the University of Sheffield in the United Kingdom.

Right next to Durrington Walls, excavators have found a village with a population that might have been in the thousands, with houses built in a style—including the placement of the beds and a central dresser—that apparently originated in far-off Orkney. Parker Pearson and others are confident that the people who lived there helped build the monuments, and the huge number of animal remains suggests that whoever was in charge of the vast project had to keep them well-fed. Other researchers have found that pottery from the village—manufactured in the Grooved Ware style first seen in Orkney—held rich traces of both dairy products and pig fat.

New evidence also supports the idea that Durrington Walls and Stonehenge served the ritual needs of a widespread



Ritual landscape. Durrington Walls (land of the living) and Stonehenge (resting place of the dead) were linked by the Avon River; other monuments, such as the Greater Cursus, were nearby.

British farmers built causewayed enclosures—deep, concentric circular ditches and banks, with narrow passageways so visitors could penetrate successive rings and reach the center. This phase lasted only about 400 years, according to the dating program, and was quickly followed around 3300 B.C.E. by another kind of earthen monument: the cursus. These linear structures are made up of two high, parallel banks with a ditch outside of each one. Cursuses are British originals, built only in these isles, though they,

too, were short-lived, in use for only about 300 years.

Up in Orkney, these earthen styles never took root, however. Instead, about 5000 years ago, the people of Orkney invented a new kind of monument—one that transformed the face of Britain.

North to Orkney

When archaeologists first glimpsed the Ness of Brodgar, after a farmer plowed up a notched stone in 2003, they had never

seen anything like it. The warren of interconnected stone buildings of various sizes remains unique in Europe, in both size and construction. To date, a team led by Nick Card, an archaeologist at the Orkney Research Centre for Archaeology (ORCA), has excavated about 10% of the site's 25,000 square meters, uncovering about a dozen stone buildings. One, thought to be a temple or meeting hall and located at the center of the complex, was 500 square meters in area and incorporated a cross-shaped interior



Celebrating the sun. The winter solstice may have been a time for rituals at Stonehenge and Durrington Walls.

population. Albarella and his Sheffield colleague Sarah Viner analyzed strontium isotope ratios in cattle teeth from the site, which vary in different geological landscapes and so can indicate where animals were raised. They found that fewer than 20 of nearly 70 tested teeth came from the chalklands around Stonehenge; the rest came from elsewhere in England and Wales. A more precise analysis using ratios of oxygen isotopes, which can reveal the location of the water the cattle drank, suggested that many came from Wales and Scotland. “Cattle will not have traveled alone,” Albarella says. “There was a gathering of people coming from many different regions, thus supporting the view of the site as potentially ceremonial.” The one human tooth found at Durrington Walls also originated far from the site, although the team can’t pinpoint just where.

These results have sparked hypotheses that far-flung hierarchies and social stratification might have been the driving forces behind the monuments. “The burials at Stonehenge might reflect some kind of royal dynasty, and Stonehenge [itself] reflect some kind of political unification,” says archaeologist Alasdair Whittle of Cardiff University in the United Kingdom.

Stonehenge and Durrington Walls might have been a unifying center for all of prehistoric Britain, or at least its southern half, Parker Pearson says. That might explain why Stonehenge’s bluestones—so named because the dol-

erite and rhyolite blocks take on a slight blue sheen when wet—were either dragged, transported on boats, or both, all the way from the Preseli Hills in Wales. Geologists Richard Bevins of the National Museum of Wales in Cardiff and Rob Ixer, now at UCL, put bluestone samples under the microscope and were able to tie them to a handful of outcrops in the hills’ northern slopes. The rhyolite bluestones came from a Preseli outcrop called Craig Rhos-y-felin, the pair suggested last year. Most of the dolerite bluestones came from another outcrop called Carn Goedog, the researchers report in a paper in press in the *Journal of Archaeological Science* and published online in November. There, bluestone pillars cling to a hillside, ripe for quarrying.

Parker Pearson and his colleagues are now excavating in the Preselis, looking for the very quarries where the bluestones began their journeys to Stonehenge, some 225 kilometers away. They conclude that the prehistoric people of Wales knew about far-off Stonehenge, and its rituals for the living, and for the dead. —M. B.



Celebrated circle. Henge monuments like Stonehenge include a circular earthen bank and ditch, with the great standing stones in the middle.

room or sanctum; Card calls it “the cathedral.” Most buildings feature walls up to 4 meters thick and internal divisions made of stones incised with mysterious “butterfly” patterns (see photo, p. 19). The whole complex was surrounded by an outer stone wall spanning up to 100 meters, almost the entire width of the isthmus. Radiocarbon dates on charcoal found under a section of this wall, and of cattle bones in one of the later buildings, indicate that the complex was first occupied about 3200 years ago and

was used for about a thousand years.

Whatever was going on at the Ness was apparently accompanied by feasting on a very large scale. In the “cathedral” building, the team found shin bones representing hundreds of cattle, deposited near the end of the life of the Ness around 2300 B.C.E. Pottery is everywhere, and unpublished analyses of residues in the pots by researchers at the universities of Bristol and York show that about half the pots contained fats from cattle carcasses, and half fats from dairy products.

These latest discoveries emphasize the importance of monuments as places where people came together to perform rituals. “The Ness was the hub of a huge wheel that took in at least all of the islands and chunks of the mainland as well,” Edmonds says. “It was the center of their universe.” Colin Renfrew, an archaeologist at the University of Cambridge in the United Kingdom, agrees. “This was a major ceremonial and ritual center that must have served for the whole of Mainland Orkney and the



Stone Age Mecca. The Ness of Brodgar was strategically located, occupying the width of a narrow isthmus between two lochs, on Orkney's Mainland Island.

whole of the islands in general. It was a very special place.”

While the Ness was still in use, the people of Orkney had another original idea: They combined earthworks and stone pillars to create the first henge and stone circle monuments, a type of megalith seen only in Britain. Strictly speaking, the term henge does not refer to the great stone circles, but to the earthwork bank lined with a circular ditch that surrounds the stones. (Stonehenge is one of the few exceptions, because its ditch is outside the bank; some purists insist that it is thus not a true henge.) Today, only four of the original 10 to 12 of Orkney's Stones of Stenness on the eastern edge of the isthmus remain. But the sockets of most of the rest, as well as the earthen bank and ditch, can clearly be seen—and they date to at least 4800 years ago, some 200 years before Stonehenge rose, according to Bayliss's preliminary reanalysis of existing radiocarbon dates. About the same time, according to new dates from optically stimulated luminescence, the people of Orkney built a second henge, the even larger Ring of Brodgar, at the north end of the isthmus.

By this time, people in continental Europe had been building stone monuments for

many hundreds of years. But when the people of Orkney expressed themselves in stone, they found a new way to do it. “Our monuments are not replicas of those in Brittany,” says Vicki Cummings, an archaeologist at the University of Central Lancashire in Preston, U.K. Stone circles are rare in France, and henges are found only in Britain.

Many archaeologists think it likely that the great stone pillars represent ancestors, arguing primarily from ethnographic examples. But Colin Richards, an archaeologist at the University of Manchester in the United Kingdom, thinks he has found other clues to the meaning of the Orkney stones. During the 1970s, archaeologists discovered that the Stones of Stenness came from at least five different sources on the island. Recently, Richards teamed up with geologists and found that the 27 remaining sandstone pillars in the Ring of Brodgar appear to come from at least seven far-flung sources up to 15 kilometers away on Mainland.

Richards surmises that different communities on the island were dragging stones to this narrow isthmus in what he sees as a delicate balance of cooperation and competition. “Brodgar was a place where differ-

ent groups were competing. It was all about dragging the stones from different places, and the ability of certain groups to mobilize labor,” he says. “Stones have never been moved on that scale before. Something is happening that has never been attempted. It's a spectacle, and people are going there to watch, as much to see it fail as to succeed.”

Card says megalith building might have been key to the development of stratified societies during the Neolithic. “Who could mobilize the most people, who could quarry and bring the biggest stones—that kind of competition encouraged the development of hierarchies.” He and others cite plentiful evidence for the growth of hierarchies across Britain beginning about 5000 years ago, including more elaborate tombs and clusters of sophisticated stone houses. On Orkney, for example, the famous stone village of Skara Brae on Mainland's west coast and a village called Barnhouse, right next to the Stones of Stenness, appear to have housed some kind of elite. And the spectacular chambered tomb of Maeshowe, clearly intended for the Neolithic upper crust, lies just 1.5 kilometers east of the Ness of Brodgar.

CREDIT: HUGO ANDERSON-WHYMARK

Celebrated ceramics

Other innovations accompanied the great monuments. The people of Orkney also invented a widespread and distinctive pottery style called Grooved Ware, with flat-bottomed vessels decorated with deep incisions (see photo, below). Recent work shows that this pottery dates back to 5100 to 5300 years ago on Orkney—before its appearance anywhere else, including Ireland, which had been considered a candidate for its place of origin.

Because pottery is usually associated with the organic remains of daily life such as charcoal and animal bones, it is easier to date than stone monuments. And because the Grooved Ware style is closely linked with henge monuments all over Britain, many archaeologists consider the pottery a more reliable chronological marker for the spread of megaliths than the stones themselves. Sometime after 2800 B.C.E., after the first megaliths on Orkney had been built, Grooved Ware shows up all over England, including at Durrington Walls, a large settlement closely associated with Stonehenge, and near three spectacular henges at Thornborough in Yorkshire, says Jan Harding, an archaeologist at Newcastle University in the United Kingdom who recently led excavations at the site.

“The idea of decorating a pot this way traveled with a wider cultural package, which included henges and standing stones,” says ORCA archaeologist Roy Towers, who leads the study of the Grooved Ware pottery at the Ness of Brodgar. “These must have been very powerful and attractive ideas.” Parker Pearson agrees: “For the first time since the arrival of farming there is a truly indigenous inspiration in architecture and ceramics and other aspects of life. ... And it was the peripheries,” rather than the mainland, “that provided the radical ideas.”

Orkney is also the source of a distinctive style of house building, found during Richards’s excavations at Barnhouse and other sites in the islands, including Skara Brae. These houses featured a central hearth, stone beds, and a stone “dresser” probably used to store household items. About 300 years later, houses with just that layout appear at Durrington Walls and other sites associated with megaliths.

Only in Orkney

Why lonely Orkney was the birthplace of all these innovations is largely a mystery, but researchers have some clues. For starters, Orkney was apparently a magnet for early cereal farmers, who could have sustained the large population needed to build the monuments. Based on the labor it took to build

the island’s megaliths, Card says, “we are looking at a population of 10,000 or more.” Although cereal cultivation arrived in Britain around 4000 B.C.E., it was quickly supplanted in most of the region by herds of cattle, sheep, and pigs, according to work published last year in *Antiquity* by archaeologists Chris Stevens of Wessex Archaeology in the United Kingdom and Dorian Fuller of UCL. But Orkney and other Scottish islands were a major exception. For example, the recently excavated settlement called Ha’Breck, on the tiny island of Wyre northeast of Mainland, yielded tens of thousands of charred barley grains, more than any other Neolithic site in Britain. There was “a significant divergence in terms of subsistence practice between the mainland and the islands,” Stevens says.

One controversial idea is that northern Scotland (including the Orkney Islands) and England were colonized by different groups of European farmers, and that Orkney might even have been colonized twice. But were these supposed European newcomers responsible for the cultural innovations on Orkney? Yes, some researchers suggest, citing the com-



In the groove. This incised pottery was invented in Orkney and spread through Britain along with megalithic monuments.

mon vole, *Microtus arvalis*. This grass-eating rodent lives all over continental Europe but is completely absent from the British Isles except for one place: the Orkney Islands. The so-called Orkney vole has long been a source of mystery, and its tiny bones have been found at many Neolithic sites on the islands.

The vole was introduced into the Orkney Islands at least 5100 years ago at sites with the earliest Grooved Ware pottery, according to work reported in *Molecular Ecology* last September. Ancient and modern DNA suggests that the vole was most closely related to either

Belgian voles or those from northern France, according to a team led by evolutionary biologist Jeremy Searle of Cornell University and archaeologist Keith Dobney at the University of Aberdeen in the United Kingdom.

The data suggest that the voles arrived too late to have stowed away with the very first farmers on Orkney, who arrived at least 5500 years ago. Rather, researchers suggest that the rodents came hundreds of years later, perhaps via a second wave of immigrants. Voles easily could have stowed away in animal fodder that farmers were bringing to the islands by boat, Dobney says. In this scenario, the encounter between new migrants and an unfamiliar environment somehow sparked dramatically new cultural innovations, including Grooved Ware pottery and the creation of magnificent megaliths.

Not everyone is convinced by the vole story. No artifacts, such as pottery, link prehistoric Belgium and the Orkney Islands, points out archaeologist Alison Sheridan of the National Museum of Scotland in Edinburgh, who finds the DNA evidence unconvincing. “The arrival of the voles should not be equated with the arrival of new farming groups,” Sheridan says.

Wherever the builders of the Orkney monuments came from, their massive creations still stir the imagination. The arrangement of monuments all along Mainland’s narrow isthmus, which links two large arable regions, suggests that it was the site of periodic processions of people from all over the island, says archaeologist Jane Downes of Orkney College on Mainland. The way the Ness completely occupies the narrowest part of the isthmus suggests to some that an elite controlled this strategic place. “You can imagine people and animals moving up and down ... this ancient route,” Downes says.

As they entered the Ness, pilgrims perhaps carried torches to light the dark, stone hallways, and they would have gazed on the red painted walls incised with engravings left by their ancestors. “People were being choreographed in movement through the Ness. Everything was hidden from view by its monumental wall,” Card says. Once they had reached the “cathedral,” they feasted on roasted cattle and performed the rituals that their culture demanded. “Then you get inside and here are all the wonders. It was like Mecca. It was the end of the journey.”

—MICHAEL BALTER

LETTERS

edited by Jennifer Sills

NextGenVOICES

Results: Enduring Ideas

What recent discovery in your field will still be remembered 200 years from now? Why?

In October, we asked young scientists to send us their field's best recent ideas. We heard from almost 200 readers. A sample of the best responses can be found below. To allow for as many voices as possible, in some cases we have printed excerpts of longer submissions (indicated by ellipses) and lightly copyedited original text for clarity. To read the complete versions, as well as many more, go to <http://scim.ag/NextGen9Results>. Follow *Science's* NextGen VOICES survey on Twitter with the hashtag #NextGenSci.

Submit Now: Science Advocacy

Add your voice to *Science*! Our new NextGen VOICES survey is now open:

If you had 5 extra hours per week to devote to advocacy for science, how would you use that time?

To submit, go to http://scim.ag/NextGen_10

Deadline for submissions is 14 February. A selection of the best responses will be published in the 4 April issue of *Science*. Submissions should be 250 words or less. Anonymous submissions will not be considered. Please submit only once.

NextGen Speaks

...THE LASER, INVENTED IN 1960, HAS REVOLUTIONIZED scientific fields from physics to medicine, enabled technological advances such as fiberoptic communication and laser eye surgery, and is part of our everyday life in items like the laser pointer and DVD players. And the laser isn't done yet. As laser technology continues to improve, we can expect to soon see lasers in other applications, which could include driverless cars, quantum communication, or optical computing. Fast forward to the year 2200. The laser is part of almost everything we do, from brushing our teeth to turning on a light bulb. Electronic components now have thousands of microlasers. Almost all fields of medicine use lasers, be it for guiding a surgery or measuring blood sugar at your doctor's office. We cannot imagine a time

without the laser, just as people in the 21st century could not imagine a time without electricity or antibiotics.

ANAND RAMANATHAN

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WITHOUT A DOUBT, 200 YEARS from now the discovery of the Higgs boson will still be remembered. It has validated a 50-year-old theory and has given us a stronger understanding of the Standard Model. It also

paved the way to work at the current model until we can be sure it describes our universe as it is. Perhaps our next step will be to prove or disprove supersymmetry, or maybe we will finally be able to identify dark matter. However, the most memorable aspect will

be the collaborative effort that went into this remarkable discovery. Thousands of scientists and engineers from over 100 different countries were part of the experiments that took place at the Large Hadron Collider.... In a time when, despite better modes of communication and technology, collaboration is not fully utilized, the discovery of the Higgs boson showed the amazing possibilities that exist when the world works together.

JAMES SHEPLOCK

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THE RECENT DISCOVERY THAT WILL BE REMEMBERED 200 years from now is the catalytic properties of RNA. Catalytic RNAs are remarkable in the way they changed our understanding of life. For instance, the resolution of the ribosome three-dimensional structure shed light on the core mechanism by which life is translated from DNA to proteins, which was also a breakthrough for developing more efficient antibiotics....The history behind catalytic RNA's discovery included notable women, such as 2009 Nobel Prize winner Ada Yonath and 2003 Wittgenstein laureate Renee Schroeder,... who haven't been neglected or treated with indifference in recognition of their cutting-edge discoveries like Rosalind Franklin was during the description of DNA structure. Hopefully, the establishment of RNA as a central molecule in parallel to DNA will not only shape our understanding of life but also open a new chapter for gender equality in the future of science.

IVAN LAVANDER CANDIDO FERREIRA

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...IN 1997, COSTANZA ET AL. SHOWED FOR the first time that the economic benefits provided by ecosystem services likely far outweigh the gross domestic product for



An exceptional
cosmic outburst

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Defense and
diversity

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the entire global economy [*Nature* **387**, 253 (1997)]. This discovery subsequently spurred an enormous amount of research and awareness for ecosystem services. Perhaps most important, this discovery challenged us as a species to take a good long hard look at the relationship between the planet's ecosystems and our way of life. If humanity still exists 200 years from now, it will be because we moved to protect and restore the world's natural capital based on the realization that without these ecosystem services, our species will be in big trouble....

ADRIAN WARD

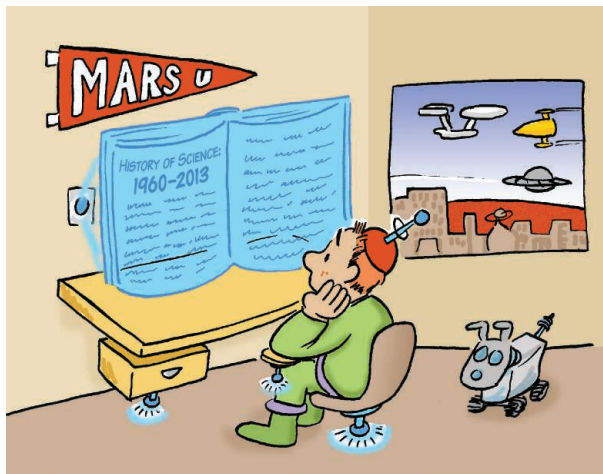
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BIOMATERIALS ARE A NEW GROUP OF MATERIALS that are nontoxic and have potential to aid diagnosis and treatment of a variety of diseases. They can be formed as porous materials that act as a scaffold for the growth of stem cells to regenerate organs for patients with disorders such as heart diseases, diabetes, burned skins, nerve injuries, and paralysis. They can be used as implants in teeth or as bone joints or replacements in different parts of the body. They can be tuned for efficient drug delivery into desired parts of the body. At nanometer sizes, some biomaterials can be injected into the body and act as contrast agents to see different parts of the unhealthy organs without doing any surgery. While the biomaterials discoveries are new, I believe that they will have a huge impact in improving human life during the next centuries.

RAZIEH KHALIFEHZADEH

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...THE ABILITY TO PINPOINT A SINGLE BRAIN cell unambiguously, subject it to investigation, and put the resulting insights into a complete context will be a landmark for



neuroscience. Like any big advance, this groundbreaking concept is a cumulative combination of techniques rather than a single point of creation....Methods to label cells distinctly provide localization and spatial information. This positional information of a cell within its network can be coupled with the power to perturb a single neuron precisely...., allowing the functional deconstruction of a highly complex organ that humans have long

found intractable....Regardless of the stage of advancement at which we might find the field of neuroscience in 200 years, the principle and ability to understand the brain's components as well as their relation to the whole will provide routes to understanding the emergent properties that are more than the sum of its parts.

EUGENE L. Q. LEE

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IN PLANETARY SCIENCES, WHAT WE WILL remember in 200 years won't be a specific discovery, but a journey of discoveries: the adventures of the Mars rovers. Following the spirit of the legendary surveyors of the Earth's borders, the Mars rovers have just started a new chapter in the history of human exploration. Only, for the first time, the voyage is on another planet. The journey

began in 1997, when the pioneer Sojourner landed on the outlet of the gigantic ancient channel Ares Vallis and demonstrated that we can rove on another planet. The MER twins first touched the surface of Mars early in 2004, with the mission of exploring



opposite sides of the planet for at least 3 months and covering maybe half a mile. They exceeded the most optimistic dreams: Spirit sent her last communication to Earth from Gusev crater in 2010, while Opportunity is still active and celebrating this week her 10th anniversary on the plains of Meridiani, after traversing over 24 miles. In 2012, the Curiosity rover started her investigations inside Gale crater, where she can potentially explore for some decades. These

missions have proven to be so revolutionary for Planetary Sciences during these pioneering years that we are already assembling the next ones, ExoMars2018 (ESA) and Mars2020 (NASA). We can expect that astronauts will be visiting the frozen remains of the rovers when humans finally set foot on Mars. Hopefully, that time will be soon enough that most of us will still be around. If not, in 200 years the Mars rovers will be waiting there, as a tribute to the human spirit of exploration....

ALBERTO G. FAIREN

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THE PROBING OF THE SUBGLACIAL AQUATIC environments in Antarctica, especially by the Whillans Ice Stream Subglacial Access Research Drilling project (WISSARD), has revealed microbes living in some of the harshest conditions on Earth. In 200 years, humans will have explored far more environments with even harsher conditions than those of Antarctica. The discovery of life in Lake Whillans allows scientists to wonder if our accepted conditions for habitability are flawed. While this project has altered our basic knowledge of life on Earth, it simultaneously permits scientists to believe in the possibility of life on other planets. We have recently ascertained more than ever about the

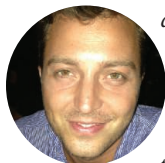


environments of our extraterrestrial neighbors, and 200 years from now this discovery will have further propelled the search for life elsewhere. Scientists have already proposed life in Mars's icy waters and on Europa, one of Jupiter's moons, based on the findings of WISSARD's research. On another note, the more we know about the different ecosystems here on Earth, the easier it will be to make decisions on how to protect them. As the icy continent is a prominent figure in the global warming frenzy, this research may provide ample reason to curb our emissions....

LAUREN C. SHAW

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...WE ARE WITNESSING THE DAWN OF A NEW era, launched by CRISP-Cas9, a known bacterial self-defense mechanism used to edit complex genomes easily and efficiently.... Editing genomes will benefit all branches of scientific research, and all organisms might easily become model organisms.... An enzymatically inactive Cas9 can inhibit the expression of a single gene [Qi *et al.*, *Cell* **152**, 1173 (2013)]. This suggests obvious translational aspects: It is possible to envision a gene therapy approach in which we target disease-causing mutations, rearrange leukemic trans-



locations, or insert or delete pieces of DNA in our stem cells or in human embryos! This discovery clearly shows our profound understanding of the several complicated components constituting the evolutionarily conserved molecular machinery within all living cells....

CLAUDIO CANTÙ

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THE DISCOVERY OF PHOTOELECTROCHEMICAL water splitting by Honda and Fujishima [*Nature* **238**, 37 (1972)] will be still remembered 200 years from now. Nowadays, rapidly diminishing fossil fuel reserves and the serious pollution caused by fossil fuel combustion are two major problems that people have to resolve for sustainable future. The solar-to-chemical energy conversion is the ultimate goal for scientists in the field of energy generation. Hydrogen from water as a form of clean energy source can provide the ultimate solutions for the energy shortage and pollution problems. Since Honda and



Fujishima's work, numerous materials have been designed and tested for photocatalytic water splitting....In the future, hydrogen derived from water splitting by renewable solar energy will serve as a clean, sustainable, feasible, and affordable energy system. In 200 years, as future people are trying to resolve their problems while sitting in their zero-pollution and solar driven vehicles, perhaps some of them will remember the work of Honda and Fujishima in 1972.

JIAN LIU

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I THINK INDUCED PLURIPOTENT STEM CELLS (iPS cells), which were generated by Shinya Yamanaka in 2006 and earned him the Nobel Prize in Physiology or Medicine in a fairly short time in 2012, will endure as a proud milestone in the history of biology.... iPS cells hold great promise for application in regenerative medicine because a patient's own cells can be induced first to be a stem cell and then to differentiate to a variety of cell lines that may be used for cell therapy and tissue and organ engineering without the concern of transplant rejection. Although there are some technical limitations and safety concerns, iPS cells will eventually fulfill their great potential to revolutionize medicine with our ever-advancing understanding of nature and developing scientific methods. In 2214, people will not only remember the first iPS cells but they will also be benefiting from them in their daily lives.



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THE SYNTHETIC CELL. THIS WAS A MAJOR breakthrough by the Venter Institute in coming out with the first synthetic cell complete with its own synthetic genome. This is the biggest demonstration of the power of synthetic biology in making living systems that are not constrained by natural forces. A sensible application of this technology will open up many possibilities such as industrial process innovation, green chemistry, and creation of new and synthetic ecosystems....



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A POPULAR SCIENCE FICTION MOVIE, *GATTACA*, envisioned a future where in utero genome profiling would determine a person's life span, health, intelligence, and any other conceivable trait. What if, in addition to understanding the genotype to phenotype relation so well, we could alter genomic makeup to achieve a specific outcome? In 200 years, the recent developments in genome engineering will not only be remembered, but become a standard procedure, like getting a vaccine. The ability to alter and control our very own genomes in such a specific way is unprecedented and will revolutionize science and medicine for years to come.



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I AM A CHEMIST, AND MY ANSWER IS A CHEMICAL material: graphene. Graphene is an ordered form of carbon in a regular hexagonal pattern; it is a single planar sheet of sp² bonded carbon atoms. Physicists at the University of Manchester and the Institute for Microelectronics Technology first isolated individual graphene planes in 2004. They also measured electronic properties of the obtained flakes and showed their unique properties. These discoveries led to an explosion of interest in graphene.... High-quality graphene is very strong and light, nearly transparent, and an excellent conductor of heat and electricity.... Graphene's extraordinary properties will make it a perfect future material; it is good for making transparent touch screens, LCD screens, and composite materials, and it can be used for terahertz technology and sensors....



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Letters to the Editor

Letters (~300 words) discuss material published in *Science* in the past 3 months or matters of general interest. Letters are not acknowledged upon receipt. Whether published in full or in part, Letters are subject to editing for clarity and space. Letters submitted, published, or posted elsewhere, in print or online, will be disqualified. To submit a Letter, go to www.submit2science.org.

HISTORY OF SCIENCE

Bounding Reasoning into Rules

Mary S. Morgan

Does “rationality” have a career? The thesis of *How Reason Almost Lost Its Mind* is that indeed it does. Or rather, as these authors from a Max Planck Institute for the History of Science, Berlin, working group recount, this is one of several historical reinventions of what it means to be reasonable or rational. This particular life history is a largely American one, and of the Cold War era, during which rationality became a subject nurtured within various overlapping strands of the human and social sciences. Fueled and amply funded by military interests, and feeding off a diet of mathematics, rationality acquired a threatening persona—lacking thoughtfulness and full of its own brash band of new technologies compared to its older parental disciplines. The macho new American “action intellectuals” of this new rationality [labeled so by historian Theodore H. White (1)] insisted that following the rules provided by these mathematized technologies defined what it meant to be rational and so gave humans the best guides to action. These rule-following forms of reasoning were perhaps one of the strongest outcomes of what have been called the cyborg sciences (2) and thus of what—broadly conceived—might be called the computer age.

The context is critical here. But the key that unlocks our understanding of this particular projection of reasoning lies in seeing the Cold War conflict not as background but as source of the materials that were fed into the scientific mill. The conflict created, in immediate and diverse ways, the problems and testing grounds for the theories and practices of rationality that formed during this period and that were designed explicitly for fighting the Cold War. It was in analyzing those problems and in creating rules for action that scientists developed the characteristics of this new, algorithmic or rule-bound, rationality. The Berlin airlift of 1948–9—flying food in daily through the Soviet blockade—offered an urgent problem for political and military

action. It was also, in practical terms, a logistical problem. The solution was bound up with the creation of operations research and linear programming, as well as computer software, and the event itself provided an exemplar for these growing specialist activities.

The perennial topic of the Cuban Missile Crisis appears here too (of course), but with a new twist as an event subject to competing analyses: the thesis of escalation based on the threat of MAD (mutually assured destruction, the game theoretic rules-based rationality for holding a large nuclear arsenal) versus the thesis of deescalation (based on an account of human reasonableness and

reasoning) found in GRIT (graduated and reciprocated initiatives in tension reduction). Humanity might be saved from the threatening and mindless rules of rationality, but those offering alternative forms of reasoning as guides to action, such as GRIT’s account based on research into human perception and trust, were branded irrational when they were

lined up against algorithmic rationality.

We gain an immediate sense of the power of Cold War rationality in events such as these, where the human sciences met each other at the point of such actions and where algorithmic rationality was used in the analysis of the crisis and its solution. It is harder to gain the same sense of what such rationality meant in other contexts that lacked this immediate need for technologies of action, even where the Cold War presence was just as pervasive. For example, we learn that there was psychological testing of whole populations on certain Micronesian islands that lay within the nuclear test site region, but we get little sense of the outcomes of that research. Just what was entailed in those peoples’ rationality? And did it matter to the bomb-testing program? How and where did these studies, and similar detailed or large-scale collections of psychological data, relate to the algorithmic rationality of the Cold War?

What is strange then about the “career of reason” in the Cold War? It is comforting that these chapters fit with our existing histories of human, behavioral, and social sciences; of the mathematical and technocratic demands and ambitions of those fields in the post-War American academy; and of the immense influence of their military patrons on the content and direction of their research in that time. The development of distinctive new elements in the human and social sciences in conjunction with mathematics and computer science

to produce cognitive science, artificial intelligence, operations research, and so forth has also been charted. So has the expansion of social science game theory into evolutionary biology. But the materials of the book are more broadly revelatory in three important ways. One relies on the connections it allows us to see and draw among actions, critical political situations, and discussions of rationality. Because of these, we can see more clearly how rule following or algorithmic reasoning lost its connection with human reasoning—even to the extent that rationality could be understood to characterize not only nonhuman activities but also activities located elsewhere than in a thinking mind, for example, in the genes of evolutionary biology. This was distinctly odd.

How Reason Almost Lost Its Mind

The Strange Career of Cold War Rationality

by Paul Erickson, Judy L. Klein, Lorraine Daston, Rebecca Lemov, Thomas Sturm, and Michael D. Gordin

University of Chicago Press, Chicago, 2013. 267 pp. \$35, £24.50. ISBN 9780226046631.



Earnest war gaming. RAND analysts engaged in a mock air and missile battle [from (3)].

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But (and perhaps this was not recognized by the book's authors) not only did algorithmic rationality become nonhuman, elements within the social sciences—particularly in economics and political science—associated with developing this notion of rationality became strangely nonsocial in the middle of the century. As these strands squeezed their focus and research onto the rationality of individual behavior, they did so at the expense of the social. Though the Cold War has collapsed and (as we see in the final chapter) algorithmic

rationality was seriously challenged, this subversion and thinning of the social into the individual remains with us into the 21st century. Over the same interval, the behavioral sciences that grew out of this Cold War experience created their own niches between (and within) the human and the social sciences.

And most important of all, and deservedly made strange for the reader: The authors show how dangerous our behavioral scientists (and by implication their human and social science kin) might have been,

co-opted as they were into the military and political decision-making in crisis situations just as physicists were co-opted into the construction of the bomb.

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EXHIBITION

Foreign Ground, Common Bodies

The Wellcome Trust, among the world's wealthiest medical charities, supports studies around the globe and funds several geographically dispersed research units. The Wellcome Collection wanted to know how the scientists who work in these research outposts and, perhaps more important, the communities in which they are embedded perceive science. So in 2012 the Collection invited several artists to serve as interpreters in year-long residencies at six of the research stations. The results of this Art in Global Health project are now on display in London.

In both art and science, trust must be established before a project can proceed. At their best, the artists' activities brought understanding and lubricated the science. Miriam Syowia Kyambi and James Muriuki set up a photo parlor in Kilifi, Kenya (site of the Kenya Medical Research Institute–Wellcome Trust Programme). They equipped their "Pata Picha Studio" with aspirational domestic props, a microscope, and a set of decorated lab coats. The thoughtful and funny resulting photographs help to correct the division between the perceptions of the scientists and their "subjects."

In turn, artists may have to overcome scientists' skepticism about the quality and value of their expertise. Katie Patterson undertook a task no less ambitious than a depiction of the evolution of life on Earth. Through her residency

at the Sanger Centre, Cambridge, she made, with the help of a Hatton Garden lapidarist, an exacting and exquisitely rendered "string of life": her interpretation of the fossil record as a necklace of stony beads on a silken thread. Artists surprised by what the scientists found beautiful turned this surprise to good effect: The B-Floor Theatre, Thailand (working with the Wellcome Trust–Mahidol University–Oxford Tropical Medicine Research Programme), and Wandering Moon Shadow Theatre company transformed potentially fatal pathogens replicating within cells into a shadow play and a brilliant set of colored transparencies. Fascinated by the human intimacies required by zoonotic infection (avian influenza in par-

Foreign Bodies, Common Ground

Danielle Olsen, curator
Wellcome Collection,
London. Through 9 February 2014.
www.wellcomecollection.org/whats-on/exhibitions/foreign-bodies-common-ground.aspx

ticular), Lêna Bui (with the Oxford University Clinical Research Unit in Ho Chi Minh City, Vietnam) created striking imagery. Her films of workers among drifting snow scenes of feathers have a serenity belied by the black dash of flies.

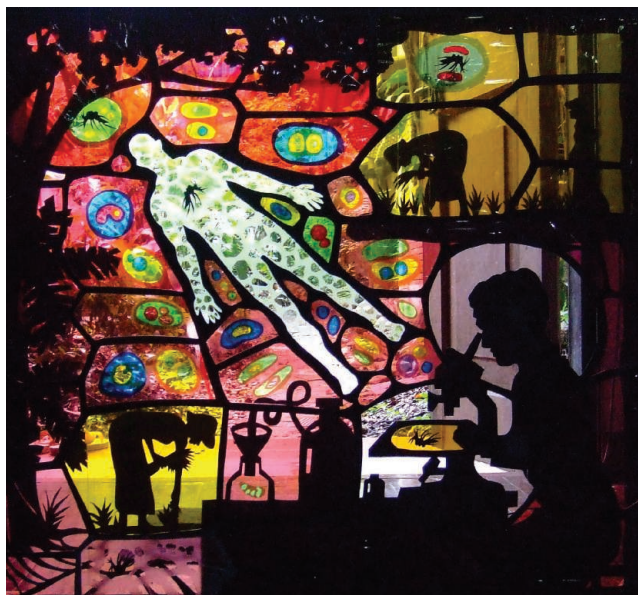
Elson Kambalu (working with the Malawi–Liverpool–Wellcome Trust Clinical Research Programme) and the Mtubatuba Workshop (based at the Africa Centre for Health and Population Studies in South Africa) were impressively inclusive in their approaches. Kambalu brought together clinicians, traditional practitioners, patients, and schoolchildren to explore the community's encounters with

medical research. People used readily available materials (such as soil, paint, glue, and flour) to create large detailed and collaborative pictures in which their main medical anxieties become obvious: the outcome of pregnancy and what scientists might use blood for—maybe witchcraft? For the Mtubatuba Workshop, South African photographer Zwelethu Mthethwa trained local people to take photographs and sent them out to depict "good health." They came back with heartening and witty portraits of a community struggling to emerge from the HIV epidemic.

The exhibition not only provides a different form of analysis for medical research. Its best works succeed because they reverse the lens on scientific activity, allowing the study participants and patients to inspect the scientists. Let's hope that the artist residencies will continue to open diverse windows of understanding between scientists and community members—who, as the show effectively demonstrates, may not always feel quite as benign about the higher calling of research as the scientists might like to think.

—Caroline Ash

10.1126/science.1249404



Wandering Moon's *Malaria and Melioidosis* shadow installation (2013).

Promoting Transparency in Social Science Research

Social scientists should adopt higher transparency standards to improve the quality and credibility of research.

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There is growing appreciation for the advantages of experimentation in the social sciences. Policy-relevant claims that in the past were backed by theoretical arguments and inconclusive correlations are now being investigated using more credible methods. Changes have been particularly pronounced in development economics, where hundreds of randomized trials have been carried out over the last decade. When experimentation is difficult or impossible, researchers are using quasi-experimental designs. Governments and advocacy groups display a growing appetite for evidence-based policy-making. In 2005, Mexico established an independent government agency to rigorously evaluate social programs, and in 2012, the U.S. Office of Management and Budget advised federal agencies to present evidence from randomized program evaluations in budget requests (1, 2).

Accompanying these changes, however, is a growing sense that the incentives, norms, and institutions under which social science operates undermine gains from improved research design. Commentators point to a dysfunctional reward structure in which statistically significant, novel, and theoretically tidy results are published more easily than null, replication, or perplexing results (3, 4). Social science journals do not mandate adherence to reporting standards or study registration, and few require data-sharing. In this context, researchers have incentives to analyze and present data to make them more “publishable,” even at the expense of accuracy. Researchers may select a subset of positive results from a larger study that overall shows mixed or null results (5) or present

exploratory results as if they were tests of prespecified analysis plans (6).

These practices, coupled with limited accountability for researcher error, have the cumulative effect of producing a distorted body of evidence with too few null effects and many false-positives, exaggerating the effectiveness of programs and policies (7–10). Even if errors are eventually brought to light, the stakes remain high because policy decisions based on flawed research affect millions of people.

In this article, we survey recent progress toward research transparency in the social sciences and make the case for standards and practices that help realign scholarly incentives with scholarly values. We argue that emergent practices in medical trials provide a useful, but incomplete, model for the social sciences. New initiatives in social science seek to create norms that, in some cases, go beyond what is required of medical trials.

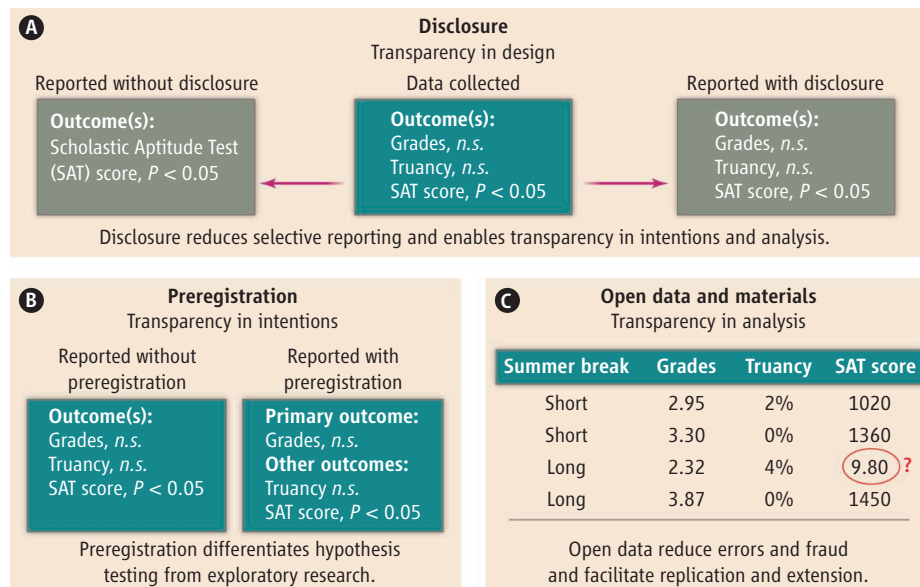
Promoting Transparent Social Science

Promising, bottom-up innovations in the social sciences are under way. Most converge

on three core practices: disclosure, registration and preanalysis plans, and open data and materials (see the chart).

Disclosure. Systematic reporting standards help ensure that researchers document and disclose key details about data collection and analysis. Many medical journals recommend or require that researchers adhere to the CONSORT reporting standards for clinical trials. Social science journals have begun to endorse similar guidelines. The *Journal of Experimental Political Science* recommends adherence to reporting standards, and *Management Science* and *Psychological Science* recently adopted disclosure standards (6). These require researchers to report all measures, manipulations, and data exclusions, as well as how they arrived at final sample sizes (see supplementary materials).

Registration and preanalysis plans. Clinical researchers in the United States have been required by law since 2007 to prospectively register medical trials in a public database and to post summary results. This helps create a public record of trials that might otherwise go unpublished. It can also serve the



Three mechanisms for increasing transparency in scientific reporting. Demonstrated with a research question: “Do shorter summer breaks improve educational outcomes?” n.s. denotes $P > 0.05$.

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purpose of prespecification in order to more credibly distinguish hypothesis testing from hypothesis generation. Social scientists have started registering comprehensive preanalysis plans—detailed documents specifying statistical models, dependent variables, covariates, interaction terms, and multiple testing corrections. Statisticians have developed randomized designs to address the problem of underpowered subgroup analysis using pre-specified decision rules (11, 12).

Open data and materials. Open data and open materials provide the means for independent researchers to reproduce reported results; test alternative specifications on the data; identify misreported or fraudulent results; reuse or adapt materials (e.g., survey instruments) for replication or extension of prior research; and better understand the interventions, measures, and context—all of which are important for assessing external validity. The American Political Science Association in 2012 adopted guidelines that made it an ethical obligation for researchers to “facilitate the evaluation of their evidence-based knowledge claims through data access, production transparency, and analytic transparency.” Psychologists have initiated crowd-sourced replications of published studies to assess the robustness of existing results (13). Researchers have refined statistical techniques for detecting publication bias and, more broadly, for assessing the evidentiary value of a body of research findings (14, 15).

Other recent initiatives create infrastructure to foster and routinize these emerging practices. Organizations are building tools to make it easier to archive and share research materials, plans, and data. The Open Science Framework is an online collaboration tool developed by the Center for Open Science (COS) that enables research teams to make their data, code, and registered hypotheses and designs public as a routine part of within-team communication. The American Economic Association launched an online registry for randomized controlled trials. The Experiments in Governance and Politics network has an online tool for preregistering research designs. *Perspectives on Psychological Science* and four other psychology journals conditionally accept preregistered designs for publication before data collection. Incentives are being created for engaging in transparency practices through COS supported “badges” to certify papers that meet open-materials and preregistration standards.

Improving on the Medical Trial Model

The move toward registration of medical trials, at a minimum, has helped reveal limitations of existing medical trial evidence (16).

Registration could similarly aid the social science community. But study registration alone is insufficient, and aspects of the medical trial system can be improved (17). The current clinical trial registration system requires only a basic analysis plan; the presence of a detailed plan varies substantially across studies. Best practices are less developed for observational clinical research.

The centralized medical trials registration system has benefits for coordination and standards setting but may have drawbacks. For instance, the U.S. Food and Drug Administration’s dominant role in setting clinical research standards arguably slows adoption of innovative statistical methods. A single registration system will have similar limitations if applied as a one-size-fits-all approach to the diverse array of social science applications. Active participation from all corners of social science are needed to formulate principles under which registration systems appropriate to specific methods—lab or field experiment, survey, or administrative data—may be developed.

Several authors of this article established the Berkeley Initiative for Transparency in the Social Sciences (BITSS) to foster these interdisciplinary conversations. Working with COS, BITSS organizes meetings to discuss research transparency practices and training in the tools that can facilitate their adoption. Interdisciplinary deliberation can help identify and address limitations with existing paradigms. For example, the complexity of social science research designs—often driven by the desire to estimate target parameters linked to intricate behavioral models—necessitates the use of preanalysis plans that are much more detailed than those typically registered by medical researchers. As medical trials increasingly address behavioral issues, ideas developed in the social sciences may become a model for medical research.

The scope of application of transparency practices is also important. We believe that they can be usefully applied to both non-experimental and experimental studies. Consensus is emerging that disclosure and open materials are appropriate norms for all empirical research, experimental or otherwise. It is also natural to preregister prospective nonexperimental research, including studies of anticipated policy changes. An early preanalysis plan in economics was for such a study (18).

Further work is needed. For instance, it is unclear how to apply preregistration to the analyses of existing data, which account for the vast majority of social science. Development of practices appropriate for existing

data—whether historical or contemporary, quantitative, or qualitative—is a priority.

Exploration and Serendipity

The most common objection to the move toward greater research transparency pertains to preregistration. Concerned that preregistration implies a rejection of exploratory research, some worry that it will stifle creativity and serendipitous discovery. We disagree.

Scientific inquiry requires imaginative exploration. Many important findings originated as unexpected discoveries. But findings from such inductive analysis are necessarily more tentative because of the greater flexibility of methods and tests and, hence, the greater opportunity for the outcome to obtain by chance. The purpose of prespecification is not to disparage exploratory analysis but to free it from the tradition of being portrayed as formal hypothesis testing.

New practices need to be implemented in a way that does not stifle creativity or create excess burden. Yet we believe that such concerns are outweighed by the benefits that a shift in transparency norms will have for overall scientific progress, the credibility of the social science research enterprise, and the quality of evidence that we as a community provide to policy-makers. We urge scholars, journal editors, and funders to start holding social science to higher standards, demanding greater transparency, and supporting the creation of institutions to facilitate it.

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Supplementary Materials

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DEVELOPMENT

The Minimalist Y

Blanche Capel

The future of the mammalian Y chromosome has been the subject of much speculation. Because the sex chromosomes, X and Y, do not recombine genetic material through most of their length during meiosis (the process that produces gametes), it is argued that the Y chromosome is degenerating, undergoing a rapid evolution that is perhaps leading it down the road to extinction (1). In contrast to this view, sequencing efforts have revealed the complex and multi-layered palindromic structure of the Y chromosome. Palindromic sequences may facilitate internal pairing of nucleotide bases, allowing the Y chromosome to repair itself through gene conversion (2, 3). This debate is among many intriguing questions about the Y chromosome and its role in gametogenesis. Yamauchi *et al.* (4) investigated the minimal number of genes from the Y chromosome that are required to produce gametes that can generate offspring using assisted reproduction methods. On page 69 of this issue, the authors reveal the answer—only two genes.

Each chromosome is divided into two “arms” by a constriction point called the centromere. It was previously shown that the entire long arm of the mouse Y chromosome (~76 Mb) could be dispensed with if the abnormal sperm produced by such mice were transferred into the egg cytoplasm by intracytoplasmic sperm injection (5). Yamauchi *et al.* show that only two genes from the remaining 2 Mb on the short arm of the Y are required for assisted reproduction in the mouse.

Previous analyses of sex chromosome variants for genes required for the various steps of spermatogenesis revealed that mice with no Y chromosome, but carrying two Y chromosome genes that were relocated to other chromosomes as transgenes, had a block in spermatogenesis—specifically, at the interphase stage of secondary spermatocytes (6). One gene was an X-linked transgene encoding *Y-encoded subunit 3 of the eukaryotic translation initiation factor 2* (*Eif2s3y*), a spermatogonial proliferation gene. The other was an autosomal transgene encoding *sex-determining region Y* (*Sry*), the male sex-determining gene. Despite the general block



Only two. Only two genes from the mouse Y chromosome, *Sry* and *Eif2s3y*, are required to produce functional male gametes that can give rise to live offspring through assisted reproductive technology.

in meiosis, some spermatids were occasionally produced. Yamauchi *et al.* used a procedure called round spermatid injection (ROSI) to show that spermatids from these mice ($X^E O Sry$) transferred into the oocyte cytoplasm can give rise to live offspring, albeit with low efficiency. This minimal complement of genes from the Y must be sufficient for all essential paternal epigenetic modifications that accompany spermatogenesis.

A block to meiotic progression occurs when the X and Y chromosomes do not pair during male meiosis (7). Pairing normally occurs through a homologous region between the X and Y chromosomes called the pseudoautosomal region. This region was missing in many of the Y chromosome variants used in the study by Yamauchi *et al.*, including $X^E O Sry$ males. The authors could rescue this problem by introducing a minute Y chromosome containing the pseudoautosomal region as a pairing partner for the X chromosome, but this did not improve the production of haploid spermatids or the frequency of ROSI-sired offspring. Most spermatids were diploid, which should have led to triploid offspring. But remarkably, diploid offspring were produced after ROSI, suggesting that ploidy was adjusted in the zygote through some mechanism not currently understood.

Further investigation of chromosome behavior and segregation at the first zygotic division of ROSI offspring from $X^E O Sry$ males should be informative.

Why not get rid of the whole Y? It should be possible to circumvent the requirement for *Sry* by overexpressing an autosomal gene like *Sry-box 9* (*Sox9*) that can drive male sex determination on its own. Although an *Sry* transcript is expressed in the adult mouse testis, it is not loaded on polyribosomes, so presumably, it is not translated (8). The testis *Sry* transcript forms a circle that serves as a “sponge” for microRNA-138, which has been proposed to be important for posttranscriptional gene regulation in the testis (9). However, overexpression of *Sox9* in a mouse that lacks *Sry* can restore full fertility (10), suggesting that the circular *Sry* transcript is not essential.

The requirement for a copy of *Eif2s3y* to drive spermatogenesis through the first meiotic division apparently can be circumvented as well. *Eif2s3y* encodes subunit 3 of the eukaryotic translation initiation factor 2. Exactly why this gene should be essential for meiotic progression is unclear. However, there is no copy of *Eif2s3y* on the Y chromosome in primates, including humans. Instead, humans have a different member of this gene

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family, *EIF1AY*, which is found in the *AZFb* region of the Y chromosome (11). Mutations in this region often lead to azoospermia (absence of sperm in the ejaculate) suggesting that *EIF1AY* may have taken over the critical function of this gene in humans (12).

Does this mean that the Y chromosome faces a bleak future? Not likely. ROSI bypasses the necessity for the differentiation steps that produce a mature sperm with a motile tail, membrane receptors that recognize the oocyte, and an acrosome that consists of a bag of enzymes that penetrate the protein shield around the egg. The short arm of the Y chromosome may encode informa-

tion essential for sperm morphogenesis, including acrosomal development, sperm head elongation, and tail morphogenesis (6). Furthermore, deletion of the gene *Sly*, which is located on the long arm of the Y, leads to derepression of other genes on the X and Y chromosomes that are normally silenced during spermatogenesis (13), and may be the cause of the sperm head abnormalities that occur in mice with long-arm deletions (14). Unless we are all planning to abandon natural fertilization and depend on ROSI to generate future generations, more of the mammalian Y will be required.

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CHEMISTRY

A Simpler Route for Making Nitrogen-Alkene Rings

Yunus E. Türkmen and Varinder K. Aggarwal

Nitrogen is a key component of many natural products and drug molecules. It has been estimated that among all natural products, the average number of nitrogen atoms per molecule is 0.7, while for medicinal drugs, this number rises to 3.0 (1). A versatile method for the synthesis of nitrogen-containing molecules is to use nitrogen-bridged alkenes (aziridines) as building blocks, as the ring strain makes them highly reactive. Aziridines are also present in a number of biologically active natural products (2) (see the figure, panel A). Current aziridination methods (3–5) almost always require the use of a protecting group on the nitrogen atom. The removal of these groups can sometimes be problematic because of the need for harsh reaction conditions. A direct aziridination method that would circumvent the need to use such a protecting group has so far remained elusive, with few exceptions (6–8). Thus, practical and direct methods for the preparation of aziridines are very useful. On page 61 of this issue, Jat *et al.* (9) report the direct synthesis of N-H and N-Me aziridines in a single step and under mild reaction conditions.

In the aziridination method developed by Jat *et al.*, the reaction of an alkene—a functional group that contains a C-C double bond—with the aminating agents DPH or N-Me-DPH [where DPH is *O*-(2,4-dinitrophenyl)hydroxylamine and N-Me denotes

addition of a methyl group onto the amine] in the presence of a rhodium catalyst affords N-H or N-Me aziridines at ambient temperature (see the figure, panel B). The success of this methodology lies in the identification of the correct combination of aminating agent, rhodium catalyst, and solvent. The initial

A common nitrogen building block used in many natural product and drug syntheses can now be made in its unprotected form in a single step.

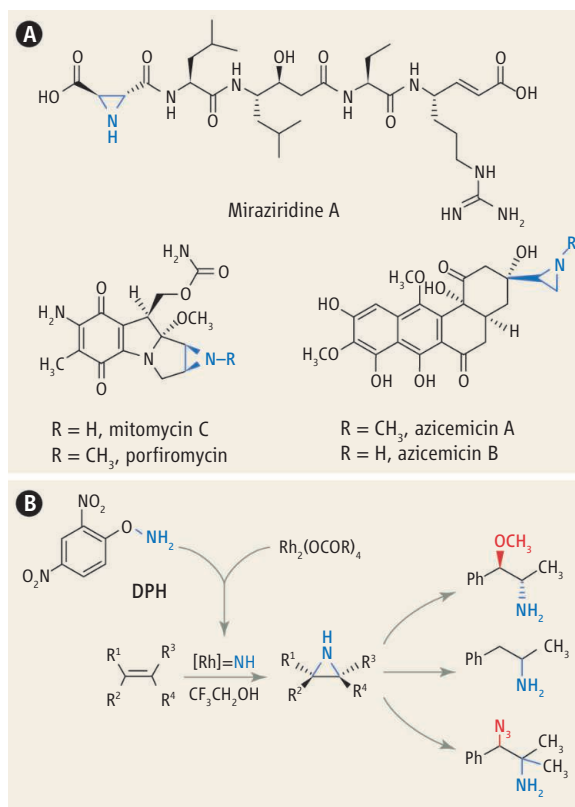
reaction of DPH with the rhodium catalyst is proposed to give a transient rhodium nitrene, a reactive intermediate with a double bond between the nitrogen and rhodium. This species is electrophilic and reacts with nucleophiles, in much the same way that opposite poles of a magnet are attracted to each

other. In the reaction mixture there are alkenes and DPH, both of which are nucleophiles, but the transient rhodium nitrene intermediate reacts selectively with the alkene reactant, resulting in the successful formation of the aziridine product.

In previous work, related aminating agents (e.g., PhINTs and TsNCINa, where Ph is phenyl and Ts is tosyl) were rendered nonnucleophilic by addition of a protecting group. Such groups not only add extra steps

Following an unprotected route.

(A) Examples of biologically active, aziridine-containing natural products are shown. (B) The reaction of alkenes with the aminating reagent DPH in the presence of a rhodium catalyst was shown by Jat *et al.* to afford N-H aziridines in a single step. Primary amines were obtained in high yields by the ring-opening reactions of the aziridine products (R groups are various alkyl groups and Ph is phenyl).



in a synthesis, but they can also be difficult to remove from the final product. By screening different catalysts, reagents, and solvents (the solvent trifluoroethanol was crucial), Jat *et al.* discovered a new, high-yielding aziridination process. Not only did the process use a low loading of catalyst, which makes it more efficient, but it also showed very broad substrate scope and high functional-group tolerance. Furthermore, the reaction preserves the two-dimensional arrangement of the groups of the starting alkene in the three-dimensional arrangement of the groups on the aziridine product. Additionally, the feasibility of ring-opening reactions of the N-H aziridine products was demonstrated in three examples affording the primary amine products in high yields (see the figure, panel B).

The new method developed by Jat *et al.* is expected to find widespread use in synthetic

organic chemistry. The aziridine products can be activated for ring-opening reactions by various Brønsted acids, Lewis acids, or other groups that are directly required in the final products (e.g., amides). This flexibility will minimize the need for functional-group manipulations and hence increase the efficiency of a synthetic sequence. Furthermore, most aziridines found in the natural products are generally in their N-H or N-Me forms, and the new aziridination protocol has the potential for synthesizing these natural products directly, without the need for deprotection of this sensitive and reactive functional group.

Many biologically active, nitrogen-containing molecules are present in single mirror-image forms (enantiomers), and because the two forms show different biological activity, it is highly desirable to make one of the two forms selectively. Otherwise, they must

be separated after synthesis. A major challenge regarding the method developed by Jat *et al.* is whether it can be rendered asymmetric by the use of a chiral catalyst, as this capability would then make the methodology even more attractive.

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ASTRONOMY

An Exceptionally Bright Gamma-Ray Burst

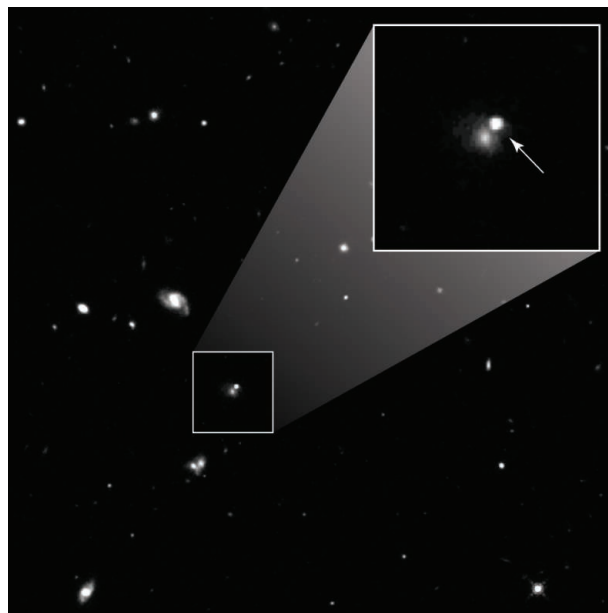
Johan P. U. Fynbo

When Tycho Brahe in November 1572 discovered a bright new star in the sky, he described it as the greatest miracle since the creation of the world (*1*). At that time, the world of stars was believed to be fixed and eternal. What Brahe had discovered was a supernova—an energetic cosmic explosion marking the end of a star's life. In this issue, four papers on pages 48, 51, 38, and 42 report on the discovery of one of the most violent cosmic explosions ever recorded (2–5). The event is a very energetic gamma-ray burst (GRB) associated with the supernova explosion of a massive star in a tiny galaxy in the constellation of Leo detected on 27 April 2013 (6, 7). A suite of satellites and ground-based observatories studied the emission from this burst at wavelengths ranging from radio waves over visible light to very high-energy γ -rays. The dataset provides an unprecedented opportunity to test and improve models of this spectacular class of cosmic explosions.

GRBs were discovered serendipitously in the late 1960s by military satellites sent into orbit to detect nuclear test explosions (8). Because the typical energies of photons emit-

ted during nuclear reactions are thousands to millions of electron volts, the satellites were equipped with γ -ray detectors. GRBs are bright flashes of γ -rays lasting from fractions of a second to several hours. They are detected

at a rate of roughly one per day from random directions on the sky (9). GRBs lasting longer than a few seconds have been firmly connected to the deaths of massive stars, those more than about 25 to 30 times the mass of the Sun (10). It is only a tiny fraction of massive stars that die in this spectacular manner (11), but because GRBs are so immensely energetic they can be seen to extreme distances (12), and, therefore, they can still be detected at such a high rate. Most GRBs originate from large distances, and hence at large redshifts. The GRB recorded on 27 April, designated GRB 130427A, is a rare instance when such a bright cosmic explosion occurred in a relatively nearby galaxy at a redshift of 0.3399 (6) (see the figure). Even though a redshift of 0.3399 is equivalent to a very large distance (the corresponding time of the explosion is 3.8 billion years ago), it is among the few percentage of smallest distances



A distant flash. An image of the galaxy from which the gamma-ray burst originated and the afterglow marked by an arrow (image from Hubble Space Telescope).

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recorded for GRBs. In addition, most of the previous GRBs detected at similar distances were much fainter than GRB 130427A. It is estimated that events like GRB 130427A will be detected only a few times per century on Earth. The relative proximity provides a rare opportunity to study such an extreme cosmic explosion in great detail and will perhaps lead to more insight than numerous studies of less spectacular events.

At the time of Tycho Brahe, astronomers relied entirely on the human eye to detect the light from celestial objects. Today, a wide range of observatories on the ground and in space allow the sky to be observed over a wide range of frequencies from low-frequency radio waves to very energetic γ -rays. The four papers describe how GRB 130427A has provided exceptionally detailed information on GRBs based on observations using many of these new observational facilities. GRB phenomenology is very rich: The collapse of the core of the dying massive star leads to the formation of a highly magnetized neutron star or a black hole. One of the main criteria necessary for the formation of a GRB is believed to be a very rapid rotation of this newly formed

compact object. Somehow this system manages to launch a very energetic, highly relativistic jet (in which the particles move at close to the speed of light) along the rotation axis of the compact object, and the prompt emission phase is believed to originate from shocks within this relativistic jet. Shortly after the jet collides with surrounding medium, creating what is known as external shocks, which gives rise to the much-longer-lasting afterglow emission phase. Both the prompt emission and the afterglow have been studied in unprecedented detail using data from the Swift and Fermi satellites as well as data from ground-based optical telescopes. The prompt emission from the burst lasted about 5 min and was recorded at a very wide frequency range from optical light (4), x-rays (2) to very-high-energy γ -rays (3, 5). The most energetic photon detected from the burst had an energy of 128 GeV. The afterglow emission was followed over several weeks from the radio band to γ -rays (2, 4, 5), and finally the associated supernova light was also detected (6).

As described in the four papers, the full data set can be reasonably well interpreted within the current theoretical framework

sketched above, but there are challenges to understanding all the details in this amazing dataset. This is good news because it leaves us with the expectation that Nature has here given us precious new clues on how to improve our models and reach a deeper understanding of the end life of massive stars.

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ECOLOGY

On Tropical Forests and Their Pests

Phyllis D. Coley and Thomas A. Kursar

B iologists have long been intrigued by the diversity of tropical forests, where 1 hectare may hold more than 650 tree species—more than in all of Canada and the continental United States. Ecological theory suggests that if species are too similar in their resource use, one will out-compete the others; hence, neighboring species must exploit different niches if they are to coexist. However, given that plants in one hectare of rainforest experience very similar physical environments, ecologists have struggled to demonstrate sufficient niche differentiation to support such high diversity (1). In addition to the puzzle of high local diversity, tropical forests also have high species richness overall. Recent studies show that interactions with pests may promote local plant diversity, accelerate plant evolution, and enhance the proliferation of species over evolutionary time.

Neighboring trees will experience similar abiotic challenges, such as the need to acquire light, water, and nutrients. It is therefore not surprising that neighbors in both temperate and tropical forests tend to have similar traits for resource acquisition (2–5). The diversity of tropical forests thus requires the existence of other niche dimensions along which species can differentiate, leading to a diversity of ways to make a living and thereby permitting coexistence. The origin and maintenance of high plant diversity in the tropics has been attributed to greater climatic stability, productivity, age, and area of the tropics than elsewhere. But these same factors also enable higher pest diversity. Biotic interactions, particularly between plants and their herbivores, may thus be stronger in the tropics (6, 7).

The evolutionary arms race between plants and their herbivores has produced a staggering array of plant defenses. For example, the leaves of a single tropical tree may contain hundreds of distinct chemical compounds that preclude attack by almost all her-

Interactions with pest species may help to explain the high plant species diversity in tropical forests.

bivore species. An infinite number of defensive combinations are possible, with each combination susceptible only to a small subset of adapted, specialized herbivores. Moreover, the extent of herbivory may depend on the defensive profiles of the neighbors. Neighbors with similar defenses could share specialized herbivores and suffer more damage, whereas a neighbor with a locally novel profile would not be vulnerable to the common herbivores and would have a fitness advantage (see the figure).

Consider, for example, *Inga*, a genus with more than 300 species, of which 45 can coexist in just 25 ha. In these as in all other tropical trees, 75% of the lifetime damage by herbivores happens during the few weeks of leaf expansion, as young leaves are tender and high in protein. To defend themselves, *Inga* leaves make a battery of chemical compounds and also have extrafloral nectaries that produce sugar to attract predatory ants as bodyguards against herbivores. Some species have normal greening, but others delay greening to minimize resources in the leaf during the vul-

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Emerging Principles for the Therapeutic Exploitation of Glycosylation

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Background: Glycoproteins and glycolipids exist as an ensemble of glycosylated variants, or glycoforms. Specific glycoforms are directly modulated by microenvironmental cues and play a key role in a wide spectrum of biological processes. Consistent with this, certain glycoforms are also prominent in various pathological conditions. These structures are either targeted by exogenous pathogens or associated with specific disease stages or, in some cases, their aberrant expression acts as a trigger to a particular disorder. An increased molecular and structural understanding of the mechanistic role that specific glycoforms play in these pathological processes has driven the development of therapeutics and illuminated novel targets for drug design.

Advances: Intervening in cellular glycosylation pathways provides a route to the alleviation of many of the symptoms of congenital metabolic disorders. Some of these same drugs also affect glycan-mediated virion assembly and offer an exciting prospect for the development of broad-spectrum antivirals against enveloped viruses. Further stages of the viral replicative cycle can be disrupted by considering their dependence on glycosylation, and this currently forms the basis of anti-influenza drugs and potential new classes of anti-inflammatories. The development of therapeutic glycoproteins has been greatly stimulated by the advances in recombinant cellular biosynthetic technologies that can produce defined glycoforms. A prominent example of this approach is the development of monoclonal antibodies with engineered glycosylation, which display enhanced *in vivo* properties. Furthermore, antibody glycosylation can also be directly modulated *in vivo*. Serum antibodies involved in autoimmunity can be inactivated by removal of their glycans by bacterial immune evasion factors, and this technology has shown great promise in preclinical studies. Glycopeptides offer intriguing possibilities in the development of anticancer vaccines given their ability to stimulate both humoral and cellular immunity. Additionally, the HIV glycan shield is proving to be an effective target for antibody neutralization and emerging targets for vaccine design and control of infection.

Outlook: Antiviral therapy looks set to have a strong glycan component in the near future. Viral protein-folding inhibition by monosaccharide analogs and glycan-epitope-dependent antibody neutralization are both promising areas. Although a successful glycan-based vaccine to cancer or HIV has yet to be realized, recent advances in both glycopeptide immunization and elucidation of the unusual features of broadly neutralizing antibodies have provided fresh impetus to these goals. Glycan engineering will continue to deliver enhanced therapeutic glycoproteins, such as antibodies, with enhanced disease modifying properties. Last, the application of bacterial enzymes that cleave antibody glycans may offer new therapeutic opportunities.

READ THE FULL ARTICLE ONLINE

<http://dx.doi.org/10.1126/science.1235681>



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ARTICLE OUTLINE

Glycosylation in Vaccine Design

Therapeutic Disruption of Glycan-Protein Interactions

Modulation of Endogenous Glycan Metabolism

Remodeling Specific Glycoprotein Glycans for Therapy

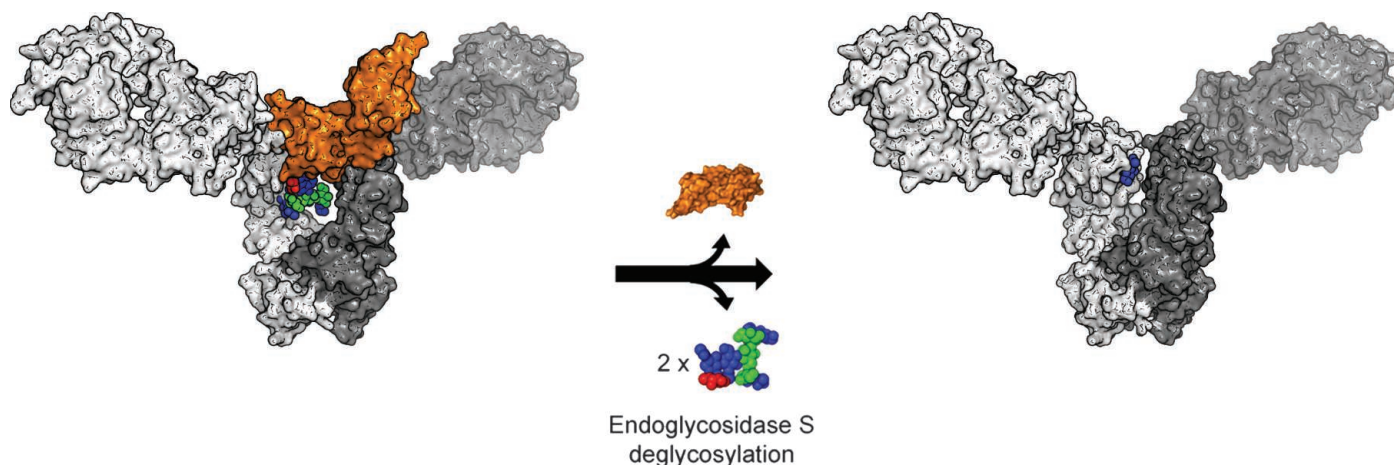
Summary

SUPPLEMENTARY MATERIALS

Movie S1

Antibody glycosylation determines Fc functions.

An example is the removal of an antibody's Fc glycans (red, green, and blue) by a bacterial immune evasion factor, endoglycosidase S, which impedes Fc engagement with cellular receptors (orange) and therefore immunological effector cells.



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Emerging Principles for the Therapeutic Exploitation of Glycosylation

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Glycosylation plays a key role in a wide range of biological processes. Specific modification to a glycan's structure can directly modulate its biological function. Glycans are not only essential to glycoprotein folding, cellular homeostasis, and immune regulation but are involved in multiple disease conditions. An increased molecular and structural understanding of the mechanistic role that glycans play in these pathological processes has driven the development of therapeutics and illuminated novel targets for drug design. This knowledge has enabled the treatment of metabolic disorders and the development of antivirals and shaped cancer and viral vaccine strategies. Furthermore, an understanding of glycosylation has led to the development of specific drug glycoforms, for example, monoclonal antibodies, with enhanced potency.

Since the term glycobiology was introduced (1), it has come to be appreciated that glycosylation is fundamental to a wide spectrum of biological processes. Advances in glycan sequencing technology have shown that glycoproteins and glycolipids exist in many glycosylated variants, or glycoforms. These can differ substantially in their biochemical properties and functions. Biosynthetic control of individual glycan structures allows both temporal and developmental regulation of glycosylation according to the functional requirements of the cell. Glycans are structurally diverse, incorporating a wide range of monosaccharide residues and glycosidic linkages. Glycans are synthesized by a large array of sequentially and competitively acting biosynthetic enzymes located throughout the endoplasmic reticulum (ER) and Golgi apparatus (Fig. 1). Mouse knockouts of these enzymes are often embryonically lethal. Knockouts can also cause selective disruptions to specific biological processes. For example, deletion of the α 2,6-sialyltransferase results in immune system dysfunction while having little effect elsewhere, whereas ablation of the Golgi α -mannosidase II chronically activates the innate immune system (2). These observations indicate that glycans are often functionally redundant outside particular biological contexts (3).

Glycans exert their biological influence in three ways. First, through their physicochemical properties that range from stabilizing protein folds to forming intrinsic components of the extracellular matrix (4, 5). Second, they are targets for recognition by glycan-binding proteins (GBPs) (6). Third, glycans can modulate the properties of the protein or lipid to which they are attached (6). Recognition of glycans by GBPs plays a central

role in cellular communication and cell trafficking (7). These interactions pervade every multicellular system and have been much explored in areas such as embryo development (e.g., Notch/Fringe) and immunology (e.g., siglecs and selectins). Within the cell itself, GBPs are used in various regulatory pathways, as exemplified by the role of calnexin and calreticulin in the ubiquitous glycoprotein-folding quality-control mechanism within the ER (8).

The functional impact of glycosylation of individual molecules is well illustrated by its effect on serum glycoproteins, where glycans can influence a glycoprotein's circulatory half-life and its functional interactions with other proteins. For example, antibody glycosylation influences antibody half-life and interaction with cellular immune receptors (9–11). Similarly, glycoforms of the red blood cell modulator erythropoietin substantially affect in vivo efficacy through their ability to directly modulate its clearance from the bloodstream (12).

In pathology, the many different roles of glycans reflect their multiple functions in both tissue homeostasis and host-microbe interactions. Their involvement either is that of targets for exogenous pathogens or involves aberrant expression that triggers or exacerbates an endogenous disease. Many pathogenic microbes make use of glycans during critical early steps in their invasion of host tissue. They achieve this through the use of various GBPs, such as adhesins, that facilitate their initial attachment to the mucosal surface (13). Indeed, terminal host glycan residues are the focal point of various invasive strategies by pathogens. For example, influenza viral particles bind directly to terminal sialic acid residues of respiratory epithelial cells (14, 15). On the other hand, some pathogens synthesize sialidases to remove sialic acid that obscures glycan motifs that the pathogen requires for mucosal attachment (16). In addition to the physical tissue invasion by bacterial pathogens, many symptoms of infection are the result of toxins produced by the bacteria themselves. Several of the most lethal forms of bacterial toxins target glycans; for example, the

cholera toxin binds a sialylated glycolipid on the surface of the host's intestinal epithelial surface (17).

Pathogens have also evolved several immune evasion strategies that exploit glycans. For example, the parasite *Trypanosoma cruzi* uses a trans-sialidase to transfer sialic acid from serum glycoproteins to its own cell surface as a means of both immunological masking of underlying epitopes and immunosuppression (18). However, a more widespread immune-evasion strategy is the direct synthesis by the pathogen of a limited repertoire of terminal glycan residues mimicking the host "self" structures. Examples include *Neisseria meningitidis* synthesis of polysialic acid, Lewis antigens by *Helicobacter pylori*, and the multiple glycolipid epitope expression by *Haemophilus influenzae*. This process of mimicry is the result of convergent evolutionary pressure on the pathogens. Similarly, certain enveloped viruses surround themselves with host-derived glycans when they exit an infected cell, contributing to viral evasion of antibody-mediated neutralization (19, 20).

In chronic disease, such as cancer, autoimmunity, and inherited disorders, aberrant glycosylation can be an effective diagnostic and prognostic marker (21). However, the precise contribution of glycan changes to disease progression or even genesis in many of these pathologies remains unclear. Nonetheless, a definitive example of the role of glycans in chronic disease can be found in congenital disorders of glycosylation (CDGs). These compose a group of rare multisymptom inherited disorders caused by defective genes directly involved in the enzymes for glycan processing or in supporting systems, such as protein trafficking. Identification of the exact pathway defects have revealed the physiological importance of glycosylation to specific processes (22). Although the incidence of CDGs is low, the recent identification of dysfunctional glycan biosynthetic enzymes in forms of congenital muscular dystrophies (23) indicates that such conditions may be more widespread than previously realized.

In cancer, a near-universal feature of tumor cells is altered glycosylation relative to the normal tissue from which they derive (24). Many observations are correlative and must be treated with caution with respect to causation. Nonetheless, some elements of tumor biology, such as metastasis and cell survival, have been linked to the presence of certain cellular glycoforms. In effect, many tumor cells make use of existing glycan-mediated mechanisms regulating cell motility and survival. These mechanisms involve expression of discrete glycan-GBP partnerships that directly influence critical parameters of tumor cell survival, such as apoptosis (25), or allow multiple cellular-extracellular matrix interactions and thus tissue migration and invasion (26). It remains unclear at what point in the tumorigenic process aberrant glycoforms are acquired. Whether glycans are involved in the neoplastic transformation, are a consequence of it, or appear as a result of a tumor's microenvironment is still debated. However, direct oncogene and tumor

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†This article is dedicated to Chris Scanlan, who passed away after a short battle with cancer during the writing of this article.

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suppressor signaling to key glycan biosynthetic genes suggests a relatively early appearance in at least some cancers (24).

In autoimmunity, aberrant recognition of glycans by both B and T cells can drive pathology. An example of an exogenous trigger of such pathology is the induction of antibodies against glycolipids in Guillain-Barré and Miller-Fisher syndromes after intestinal *Campylobacter jejuni* infection (27). The *C. jejuni* lipopolysaccharide contains terminal glycan motifs that resemble self terminal glycan structures. Antibodies produced by the immune system in response to infection not only bind to the lipopolysaccharide structures but can also cross-react with self glycolipids containing these motifs. This cross-reactivity can cause damage to the peripheral nervous tissue where these glycolipids are found. Antibodies against

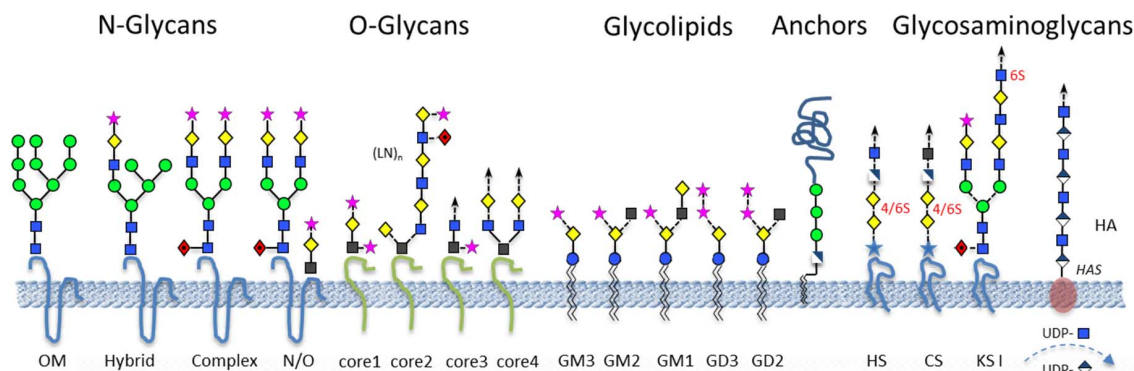
glycolipids have also been identified in a wide range of autoimmune conditions, including multiple sclerosis and systemic lupus erythematosus.

Cellular presentation of glycopeptide and glycolipid antigens can be potent modulators of T cells and thereby contribute to autoimmune pathologies (28). In multiple sclerosis, autoreactive T cells have been identified that are restricted to brain sulfatides presented by CD1a proteins of dendritic cells (29). The CD1 family is expressed by antigen-presenting cells and displays self and exogenous glycolipid and lipopeptide antigens to a range of T cells. Glycopeptide antigens can be presented by the major histocompatibility complex (MHC) system and contribute to autoimmunity. Changes in glycosylation can influence tolerance either by constraining glycopeptide presentation in the MHC system or by creating neo-antigens

(28). An example of the latter is the induction of rheumatoid arthritis by a glycopeptide fragment of type II collagen (30) in which an O-galactose at a hydroxyl-lysine is critical to T cell recognition (31).

Glycosylation can also influence autoimmunity by modulating the activity of key regulatory components. For example, immunoglobulin G (IgG) antibody-mediated inflammation is associated with specific antibody glycoforms (32). These autoantibodies form immune complexes (IC) with self-antigens that in turn drive inflammation via recruitment of complement and effector cells, subsequently leading to localized tissue damage. Specific IgG Fc glycoforms (Fig. 2) are associated with IC formation, binding of activatory IgG Fc γ -receptors (Fc γ R), complement activation, and severity of inflammatory response in various autoimmune diseases, including rheumatoid arthritis.

A Major classes of glycan



B Biosynthesis

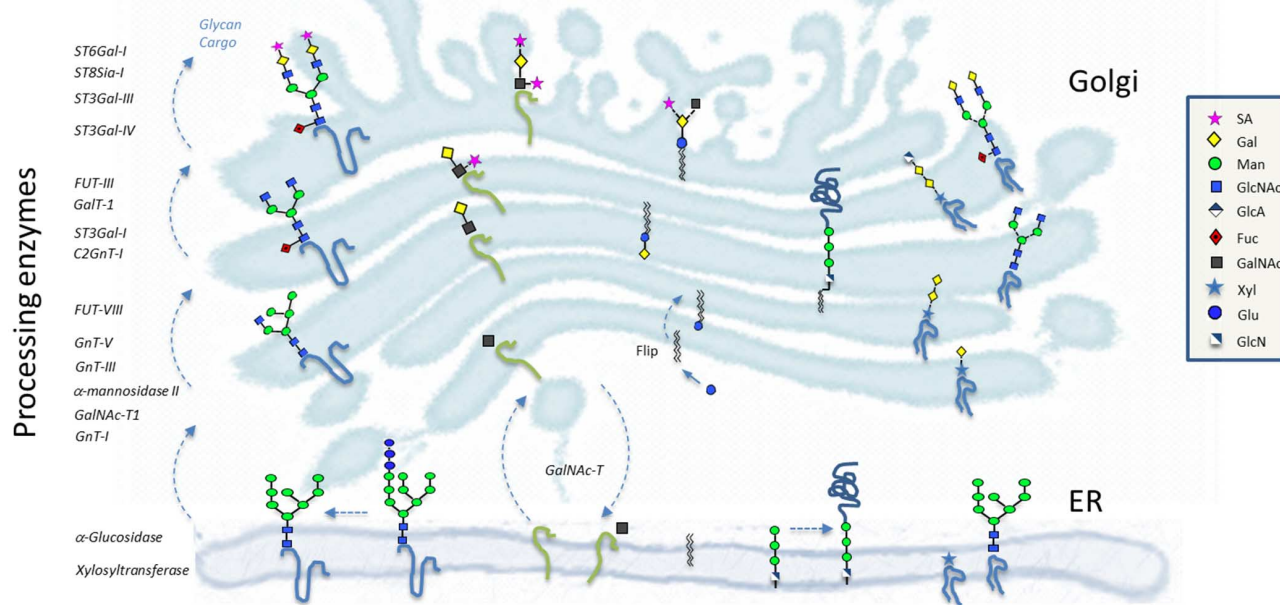


Fig. 1. Mammalian glycan biosynthetic pathways. Schematic depicting (A) the various classes of glycans (CS, chondroitin sulphate; HA, hyaluronan; HAS, hyaluronan synthase; HS, heparin and heparin sulphate; KS, keratin sulphate; GM and GD, mono- and disialylated glycosphingolipids, respective-

ly; OM, oligomannose) and (B) their associated biosynthetic pathways in the ER and Golgi apparatus (6). Monosaccharide abbreviations are as follows: Gal, galactose; Glu, glucose; GlcA, glucuronic acid; GlcN, glucosamine; Fuc, fucose; SA, sialic acid; Xyl, xylose.

Conversely, fully sialylated IgG Fc seems to promote an anti-inflammatory response (9).

In summary, the above insights, highlighting the pivotal role played by glycans in various human pathologies, are now driving efforts toward their medical exploitation (Fig. 3).

Glycosylation in Vaccine Design

Effective vaccines should elicit a robust IgG response with associated memory B cells. Although pathogen-induced antibodies against glycans (anti-glycans) are primarily low-affinity IgM, potent IgG responses can be achieved. This is typified by our high-affinity IgG response to the nonhuman α -galactose epitope, which is a major obstacle to xenotransplantation. Indeed, this antibody-mediated tissue rejection initially gave impetus in the development of antiglycan vaccination strategies against tumors (33).

To circumvent the issue of poor immunogenicity, glycan-based vaccines need to be conjugated to helper T cell epitopes such as keyhole limpet hemocyanin (KLH). Synthetic chemistry has greatly aided this process by the use of specific linkers that do not disturb the immunogenicity of the glycan being conjugated. Coupling synthetic conjugation with synthetic carbohydrate synthesis using techniques such as one-pot and automated oligosaccharide synthesis has greatly

facilitated both vaccine design and testing (34). New conjugation technology aimed at boosting immunological efficacy is also in development. Examples include novel T cell epitopes, Toll-like receptors, and tetanus toxoids (35). One fascinating variant is the direct conjugation of target antigen to the α -galactose epitope that exploits our zoonotic blockade of this motif (36). Last, the discovery that glycopeptides can be presented by the MHC system has also opened up the possibility of generating cytotoxic T cell responses toward tumors. Vaccination that induces the effector functions of both humoral and cellular immune systems are currently being investigated (34).

Cancer vaccines based on tumor-associated glycans have received a significant amount of attention, and the epithelial mucin, MUC1, has been at the forefront of this work. MUC1 is both overproduced and aberrantly glycosylated in many forms of cancer (37). This abnormal mucin contains glycopeptide epitopes (e.g., O-glycan T, sialyl-Tn, and Tn) that can be immunogenic. Patients who mount a significant response to MUC1, particularly its glycan component, have a more favorable prognosis (38).

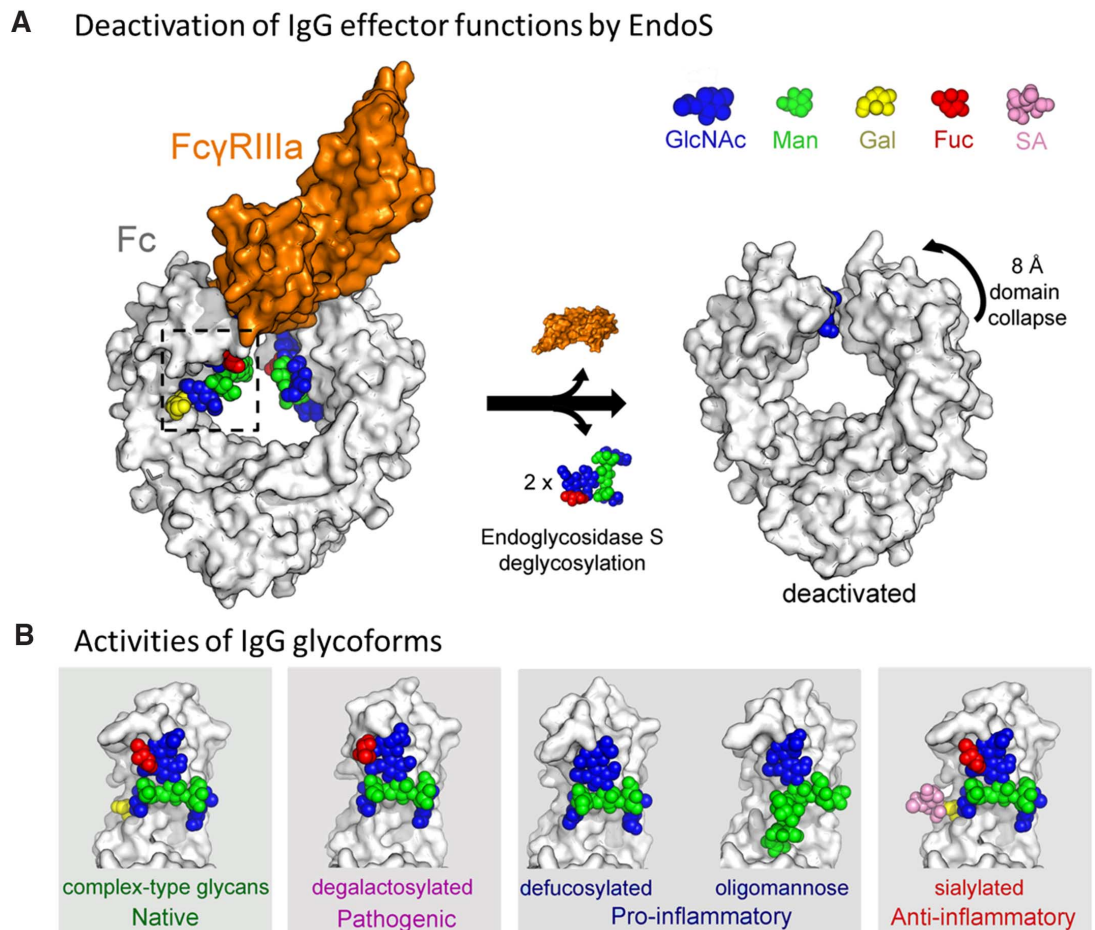
Attempts to elicit an effective anticancer immune response to MUC1 have exploited glycopeptides based on variable-number tandem-repeat peptides containing the O-glycan-rich region of

MUC1. The MHC presents MUC1-derived glycopeptides containing cancer-associated Tn epitopes with greater efficiency than the unmodified peptide (39). Glycopeptides seem to elicit significant cellular responses and anti-glycan-mediated antibody-dependent cellular cytotoxicity (ADCC) (40). Multiple cancer vaccine trials are now in progress using specific synthetic MUC1 peptides and glycopeptides of varying peptide lengths and cancer-associated glycan structures (37, 40).

However, the expression of cancer-associated epitopes can vary dramatically in different patient groups. This may have contributed to the conclusion that a sialyl-Tn/KLH vaccine did not impart significant survival benefit in a double-blind randomized phase III trial of 1028 women with metastatic breast cancer despite good seroconversion, specific IgG anti-sialyl-Tn titers, and promising phase II trials (41).

Sialylated glycolipids, termed gangliosides, have attracted considerable interest as potential targets of immunotherapy. However, vaccination strategies against gangliosides GD2 and GD3 have yet to yield positive clinical data (42). In contrast, the use of “nonself” tumor-associated gangliosides, such as *N*-glycolylneuraminic acid (Neu5Gc)–containing GM3, has yielded promising data (43). Neu5Gc is a modified sialic acid not made by humans because of a defective hydroxylase gene

Fig. 2. Antibody glycan engineering. (A) Structure of the Fc-FcγRIIIa complex and that of the deglycosylated Fc after cleavage by EndoS (90, 97). Deglycosylation reduces receptor binding and is being investigated for the treatment of autoimmune diseases (32, 92, 93) and the production of therapeutic deglycosylated antibodies (95, 96). (B) Crystal structures of different glycoforms exhibiting a range of effector functions (89, 98–100).



(44). Tumor-associated Neu5Gc is derived from the diet and is thought to promote localized weak chronic inflammation (45), which is now recognized as a necessary hallmark of cancer (46). There are currently two phase III vaccine trials taking place based on Neu5Gc including racotumomab (47), a murine monoclonal anti-idiotype.

Another prominent role for glycans in vaccine design is found in some immunotherapeutic approaches toward neutralizing enveloped viruses. HIV best exemplifies this field, where glycans constitute about half the mass of its attachment glycoprotein, gp120. Although the trimeric HIV gp120 attachment and the gp41 fusion spike are virally encoded, the glycan component is synthesized by the infected host cell. These glycans protect the virions from antibody neutralization. However, their very high density leads to impaired glycan processing and the generation of a population of oligomannose-type glycans, which are typically present at very low levels on cell-surface and secreted glycoproteins (48).

The viral glycan shield offers an attractive vaccine target because it is distinct from typical self-glycosylation. It is largely structurally conserved across clades relative to the underlying protein sequence (49), and broadly neutralizing antibodies (bnAbs) have been identified from some individuals that recognize the glycan shield (50). These features, together with the observation that passive transfer of these bnAbs offers significant protection to infection, suggest that a successful HIV vaccine may well have an anti-glycan component.

The first anti-glycan shield bnAb identified was IgG1 2G12, which is effective against a relatively broad range of antigenically unrelated HIV-1 isolates with the notable exception of the epidemiologically crucial clade C (51). Although 2G12 exhibits an unusual domain-exchanged structure (52), many other bnAbs (such as the PGT and the PG series) have now been isolated that conform to a more typical IgG structure and exhibit a wide neutralization breadth and potency (50). Unlike the epitope of 2G12, which is entirely oligomannose-type glycans, the PGT and PG series have a mixed glycan-peptide epitope (53–55). In the case of PGT128, the breadth of the protein recognition is maintained by exploiting contacts with the peptide backbone.

The emergence of a large range of clonally unrelated bnAbs that recognize gp120 around the conserved N-glycan site at Asn³²² (N332) raises the question of how different, unrelated antibodies can recognize the same site of vulnerability on the virion surface (55). The structures of 2G12, PGT128, and PGT135 in complex with outer domain fragments show highly distinct modes of binding, suggesting that there are multiple immunological solutions to the recognition of the glycan shield (Fig. 4). Furthermore, individual antibodies demonstrate substantial plasticity of recognition and tolerate some diversity in both the glycans recognized and shifts in the glycan location (56). One might argue that plasticity in recognition would lead to a greater likelihood of antibodies to

this region being elicited by vaccination. However, an alternative view would argue that the lack of a single binding mode presents a challenge to rational vaccine design.

Can bnAbs against the glycan shield serve as the basis for a rational vaccine design in HIV? Glycan-binding antibodies can be elicited in experimental models such as macaques infected with a pathogenic R5 strain of simian SIV_{ADA8} (57) and yeast mannan immunization studies (58). Therefore,

although many hurdles remain, it is certainly conceivable that an anti-glycan immune response will be an important component of a successful vaccine.

Therapeutic Disruption of Glycan-Protein Interactions

There is a wide spectrum of therapeutic opportunity that has arisen from the detailed knowledge of glycan-protein interactions in both the endogenous signaling systems and the life cycles of

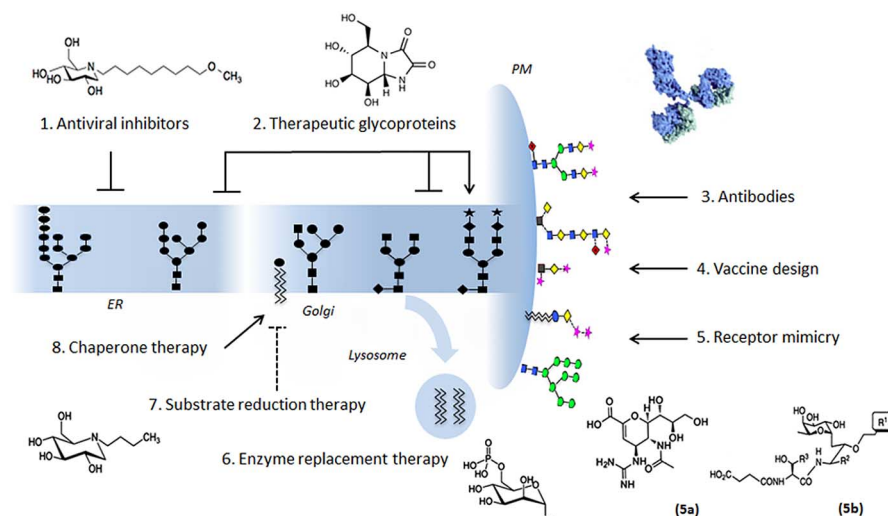


Fig. 3. Schematic representation of the major points of therapeutic intervention of glycosylation. ER/Golgi/plasma membrane (PM) glycosylation processing is shown with major intervention strategies indicated. Specific structures indicated are as follows: category 1, UV-4 (*W*-9-methoxynonyl DNJ); 2, kifunensine; 3, IgG; 5a, carbohydrate mimetic selectin inhibitor; 5b, Relenza; 6, M6P; 7, NB-DNJ (miglustat).

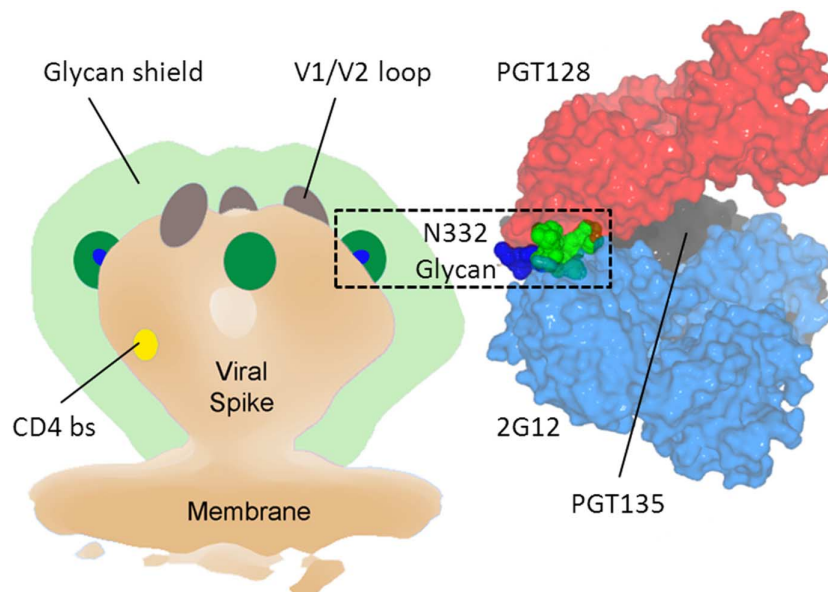


Fig. 4. Antibody recognition of the glycan shield of HIV. Schematic representation of the viral spike of HIV based on cryogenic electron microscopy (beige) with the glycan shield (green) (56). The position of the glycan at N332 is shown together with the crystal structures of the Fabs of PGT128 (red), PGT135 (gray), and 2G12 (blue) (52, 54, 55). These antibodies recognize more than one glycan and, with the exception of 2G12, also bind the underlying protein. Further information on the relationship between gp120 glycans and 2G12 recognition is available in video format in the supplementary materials (movie S1).

pathogens. This therapeutic area draws extensively on synthetic chemistry and emerging technologies, such as phage display, for the design and construction of high-affinity inhibitors (59, 60).

Many pathogens rely on specific glycan sequences as part of their infection strategy, and this has led to the concept of using these or their derivatives to impede infection. Small-scale trials have been reported that used free glycans to inhibit a range of microbes as well as several pathogenic viruses (17). Such receptor mimicry strategies often need to overcome the large avidity of the natural receptor generated by the sum of multiple presented low-affinity glycan binding sites. An example of a successful strategy that takes avidity considerations into account is the inhibition of shiga-like toxin of the *Escherichia coli* strain O157:H7 using a multimeric compound that has numerous glycan termini (17). Low-affinity glycan-protein interactions are also being targeted in development of anti-inflammatories, which antagonize selectins that mediate leukocyte extravasation through the recognition glycan epitopes such as sialyl-Le^x. Although directly mimicking sialyl-Le^x results in poor inhibition because of low affinity, this structure has been used as a lead candidate in the synthesis of higher affinity mimetics (59).

Synthetic mimics of glycans are also used as antivirals. Two of the four approved drugs against the influenza virus, zanamivir (Relenza, GlaxoSmithKline) (15) and oseltamivir (Tamiflu, Genentech) (14), are directed against the viral envelope glycoprotein neuraminidase, an enzyme that enables the release of viral particles from infected cells by cleaving sialic acid on the cell surface. Knowledge of its crystal structure led to the development of the drug Relenza, which is specific against influenza and does not inhibit mammalian neuraminidases (61). Tamiflu was developed as an orally available neuraminidase inhibitor, based on Relenza. Although some chemical features of Relenza were maintained, a range of modifications aimed at formulating it as a pill reduced its structural similarity to the natural substrate, a factor that may have contributed to the comparably faster emergence of viral escape mutants against this drug.

Faced with high mutation rates of viral target proteins, direct acting antivirals generally present low genetic barriers to resistance. However, targeting host cell pathways to raise the genetic barrier for viral escape mutants would circumvent this problem and potentially provide broader acting antivirals. Viruses rely on host cell processes, such as glycosylation, to enable cellular uptake, replication, secretion, and spread of infectious particles. For rapidly mutating RNA viruses like hepatitis C virus (HCV), HIV, influenza, and dengue, the concept of a generic therapy capable of dealing with current master species, as well as existing and potential mutants and reassortants, is appealing. Antivirals that could target several different virus species simultaneously could provide better treatment options for the increasing numbers of coinfecting patients.

The eukaryotic ER has a protein quality-control machinery that monitors and enables the

correct folding of nascent glycoproteins (8). After the sequential trimming of terminal glucoses attached to N-glycans by ER α -glucosidases I and II, the interaction of monoglucosylated glycoproteins with calnexin and/or calreticulin prevents folding intermediates from premature export, aggregation, or destruction. Once ER α -glucosidase II removes the final glucose residue, glycoproteins are liberated from the calnexin/calreticulin cycle. If the protein has failed to reach its native conformation at this point, it will be recognized by uridine diphosphate (UDP)-glucose glucosyltransferase, which transfers one glucose residue from UDP-glucose to regenerate Glc₁Man₉GlcNAc₂, (where Glc is glucose; Man, mannose; and GlcNAc, N-acetylglucosamine) restoring recognition by calreticulin and calnexin.

Preventing the formation of mature, infectious virus by inhibiting the glycoprotein-processing pathway provides an attractive target for the development of broad-spectrum antivirals. Any virus that depends on calnexin-mediated folding of their envelope glycoproteins is vulnerable to this approach, and inhibitors of ER α -glucosidases, such as the iminosugars, have demonstrated in vitro antiviral efficacy against representative targets from nine viral families: *Herpes*-, *Hepadna*-, *Flavi*-, *Toga*-, *Rhabdo*-, *Arena*-, *Orthomyxo*-, *Paramyxo*-, and *Retroviridae*. Subsequent in vivo trials in small-animal disease models have shown higher than expected efficacy, notably for those viruses causing acute disease and featuring a pronounced cytokine involvement in disease progression, like influenza and dengue virus treated with *N*-9-methoxynonyl DN/(UV-4) (62). A direct effect of ER α -glucosidase inhibition on (glycosylated) cytokines or their receptors needs to be evaluated. For viruses causing chronic disease, prevention of emergence of viral escape mutants when treating for many weeks or months is an anticipated and desired aspect of developing host cell-based therapeutic strategies. For HIV and HCV, such resistance-proof treatment has been reported in vitro (63).

Modulation of Endogenous Glycan Metabolism

Congenital defects in glycoprotein and glycolipid metabolism are increasingly treatable by circumventing the metabolic blockade using dietary supplementation of required monosaccharides, administration of required enzymes, specific enzyme inhibition, or chaperone rescue.

Of all the CDGs described to date (22), only two are currently treatable to some extent by dietary supplementation (substrate replacement therapy): Mannose supplementation relieves some symptoms in mannose-6 phosphate isomerase-CDG, which is caused by mutations in phosphomannose isomerase (64–66). In another CDG, a guanosine diphosphate (GDP)-fucose transporter deficiency strongly reduced fucosylation of cell surface glycoproteins (64). This prevents selectins involved in leukocyte extravasation from binding, resulting in leukocytosis and increased sensitivity to infections. An intriguing finding is that the

defective Golgi GDP-fucose transporter is not required for the therapeutic effect of exogenous L-fucose. The identity of an alternative transport system that would explain this conundrum remains elusive (64).

Enzyme replacement therapy (ERT) has emerged as an effect treatment for lysosomal storage diseases (LSDs). LSDs are caused by genetic mutations that result in defective enzymes affecting the activity or trafficking of lysosomal hydrolases. Gaucher patients suffer from a deficiency of the enzyme glucocerebrosidase, and, for severely affected patients, replacement of the deficient enzyme by intravenous infusion with recombinant analogs imiglucerase (Cerezyme, Genzyme) and velaglucerase (GA-GCB and VPRIV, Shire Human Genetic Therapies) is an effective treatment.

The generality of ERT is also shown by the use of α -galactosidase derivatives for treatment of Fabry disease (67–69) and the use of α -L-iduronidase (laronidase, Aldurazyme, Genzyme) (70), iduronate-2-sulfatase (idursulfase, Elaprase, Shire Human Genetic Therapies), and glycosaminoglycan *N*-acetylgalactosamine 4-sulfatase (acrylsulfatase B, Naglazyme, BioMarin Pharmaceutical) to overcome enzyme deficiencies in various mucopolysaccharidoses storage diseases (71).

Monosaccharide analogs that partially inhibit specific biosynthetic enzymes are also the basis of several clinical treatments. The glucose analog *N*-butyldeoxynojirimycin (NB-DNJ) is an iminosugar that reversibly inhibits ceramide glucosyltransferase. This latter activity is the basis for its approval as an alternative approach to ERT in type I Gaucher's disease, sometimes referred to as substrate reduction therapy. The ability of this iminosugar to cross the blood-brain barrier enables the treatment of LSD with neurological manifestations and is approved for the management of neurogenerative Niemann-Pick disease type C (72).

Inhibitors of α -glucosidase are also being evaluated as an alternative therapeutic approach in the treatment of noninsulin-dependent diabetes mellitus (73). The iminosugar miglitol is a clinically approved treatment that works by inhibiting the α -glucosidase hydrolase enzyme sucrase-isomaltase located in the brush border of the small intestine. This impairs carbohydrate digestion and slows glucose absorption into the blood stream, reducing postprandial hyperglycemia. Other structures under evaluation include triterpenes hybrids, pseudo-carbohydrates (acarbose and voglibose), and non-carbohydrate mimetics (73).

A seemingly paradoxical role of glucocerebrosidase inhibitors such as iminosugars is that of pharmacological chaperone therapy in the treatment of LSDs. Low doses of iminosugars such as isofagomine (Plicera, Amicus Therapeutics) partially rescue lysosomal ceramide glucosyltransferase activity in type I Gaucher's disease (74). The most accepted explanation for this mechanism is that the reversible binding of the inhibitor to the misfolded enzyme in the ER enables sufficient folding around its active site to rescue it

somewhat from degradation and allow it to traffic to lysosomes.

Remodeling Specific Glycoprotein Glycans for Therapy

Many therapeutic proteins are glycoproteins, and, although some are purified from natural sources, the majority are recombinantly expressed. The choice of expression system heavily influences the glycosylation. There have been notable efforts in controlling the glycosylation of glycoprotein production systems motivated by the potential immunological properties of nonself glycans, the impact of glycosylation pharmacokinetics, and the *in vivo* functionality (75).

The use of nonhuman mammalian cell lines has the potential to introduce immunogenic epitopes such as α -galactose and Neu5Gc (76), although Neu5Gc can arise in any mammalian system when Neu5Gc is present in the media. In plants, xylose and core N-glycan α 1,3-fucose epitopes can arise, which are known immunogenic epitopes. Insect cell lines can also exhibit α 1,3-fucose and naturally exhibit extensive mannose-terminating structures. Last, yeast N-glycans are typically extended immunogenic mannans. These features are important consideration in the production of biologicals. For example, the presence of α -galactose epitopes on cetuximab, a monoclonal from a murine cell line for use in metastatic colorectal cancer, has led to anaphylaxis in some patients (77).

Despite these observations, nonhuman cell lines such as Chinese hamster ovary (CHO) cells (which remain the industry standard), baby hamster kidney cells, murine myeloma, and murine hybridoma cell lines are prevalent production systems (76). However, the desire to control glycosylation and use a wider spectrum of nonhuman production systems has motivated important advances in glycoprotein engineering. Beyond the large panel of CHO cell glycosylation mutants that has been developed by mutagenesis and lectin selection, transgenically altered CHO cells enable both the elimination of potentially hazardous glycosyltransferases and the fine-tuning of glycosylation to manufacture desired glycoforms.

A range of methodologies has been established to remove the fucose of anticancer therapeutic monoclonal antibodies (mAbs) (78, 79), including use of stable cell lines deficient in the α 1,6-fucosyltransferase; cell lines deficient in GlcNAc transferase-I, which stalls the biosynthesis of the glycans at Man₅GlcNAc₂; and inhibitors such as kifunensine, which can trap the N-glycans in afucosylated oligomannose glycoforms (17). Furthermore, consideration of the effect of sialylation on pharmacokinetics has led to optimization of the sialylation machinery for the production of highly sialylated glycoproteins (e.g., erythropoietin).

Plants display a remarkable tolerance toward manipulation of their intrinsic glycan biosynthetic pathways (80). This has allowed the removal of potentially immunogenic residues, and the addition of appropriate extension enzymes (e.g., β 1,4-galactosyltransferases), alongside the more chal-

lenging terminators such as α 2,6-sialyltransferase, has allowed a fine-tuning of the desired glycan population (80).

Yeast glycosylation machinery has been extensively remodeled, exemplified by work on *Pichia pastoris*, yielding a panel of cell lines that offer the capacity to generate different glycoforms (81). Interestingly, because this system often has significantly elevated glycosyltransferase activity, it has been possible to manufacture antibodies with a different range of complex-type glycans beyond the natural protein-imposed biantennary type, giving access to a portfolio of effector functionality beyond that which can be achieved by mutation alone (82).

Another approach uses chemoenzymatic methods to remodel the protein glycosylation after expression (75). This approach enables chemical control of glycosylation. The control potentially afforded by this approach could have an important impact, for example, in the fine-tuning of antibody effector functions (83).

Glycan engineering has also led to advancements in enzyme replacement therapies. Substantial mechanistic challenges in ERT treatment of LSD are targeting the affected cells or tissue and delivering the enzyme intracellularly to the lysosomes. Recombinant enzymes can be produced in CHO cells and their glycans enzymatically remodeled to include mannose-6-phosphate (M6P), critical for their targeting to lysosomes via the M6P receptor (84). A mannose-terminating glycoform also helps other issues such as macrophage targeting (e.g., in Gaucher's disease). Uptake is mediated by mannose receptors, and transport across the blood-brain barrier is boosted through interaction with M6P/insulin-like growth factor 2 (85). Other expression systems are currently being explored, including human fibrosarcoma cells, egg whites, transgenic animals, and plant cells (86).

mAbs offer great promise in the treatment of disease by their ability to specifically bind targets through the Fab domains while also possessing the ability to recruit the cellular and humoral immune system through the Fc domain. The glycosylation of IgG, the predominant class of antibody used therapeutically, modulates the binding to the Fc γ R family of cellular immune receptors (87). Although Fc glycans are critical to many antibody effector functions, receptor binding can be restored by the compensatory addition of Fc amino acid substitutions (88). However, to date the majority of monoclonals under clinical trial consideration are glycosylated.

In the case of cellular targets such as cancerous cells, the immobilization of antibody across a cell surface flags the cell for immune recognition, even in cases where the primary mode of action could be ascribed to the physical mAb binding, such as the blocking of a growth factor receptor. In addition, even mAbs that target soluble ligands, such as the growth factors, can rely on their Fc domains because it is through Fc γ R recognition that the growth factors are cleared for destruction. Given the importance of Fc-Fc γ R interactions, augmenting the binding of the Fc domain to the cellular Fc γ Rs can enhance the efficacy of many proinflammatory antibodies.

The IgG Fc glycans are typically of the biantennary complex type, exhibiting high levels of fucosylation of the core GlcNAc residue, partial galactosylation, and bisecting GlcNAc. Of these structures, about <20% are sialylated. The reason for the low levels of branching and terminal structures are the constraints on glycan processing imposed by the protein. The two opposing glycans within the homodimeric Fc lie along the surface of the C γ 2 domain, and they form extensive hydrophobic interactions with the apolar faces of the saccharide residues (Fig. 2). These interactions are maximal upon biosynthetic conversion from oligomannose and hybrid-type structures to the complex type (89). Importantly, the fucose residue is highly solvent exposed in the unliganded form but clashes with the glycan of one of the key receptors, Fc γ RIIIA, the main Fc γ R of natural killer cells. This clash reduces Fc γ RIIIA binding and limits ADCC of fucosylated antibody glycoforms (90).

Glycosylation can also contribute to the anti-inflammatory properties of antibodies. High-dose (1 to 2 g/l) intravenous administration of IgG, known as IVIg, is an effective therapeutic treatment for autoantibody-mediated inflammation (10). IVIg is prepared from plasma derived from large numbers of healthy donors. An anti-inflammatory component has been identified as a subset of this IgG population bearing α 2,6-sialylated Fc glycoforms. Indeed, the efficacy of sialylated Fc provides a route to the clinic of recombinant immunoglobulin-based anti-inflammatories (9). The precise anti-inflammatory mechanism of sialylated Fc remain a subject of debate (91).

Last, enzymatic deglycosylation of IgG provides several therapeutic opportunities. Deglycosylation of IgG significantly decreases binding of antibodies to Fc γ Rs. Although several endoglycosidases have been discovered capable of cleaving the Fc glycans (Fig. 2), only endoglycosidase S (EndoS) is uniquely specific to IgG (92). This allows the possibility of selective deglycosylation of antibodies *in vivo* to treat patients with antibody-mediated autoimmunity (93). In addition to specificity for IgG at the protein level, EndoS only cleaves biantennary glycans (83). EndoS is secreted by *Streptococcus pyogenes* as part of its immune evasion strategy. Perhaps as a consequence of its effect on antibodies, EndoS does not appear to be significantly immunogenic in animals. EndoS has shown promise in the treatment of variety of autoimmune conditions, including systemic lupus erythematosus (94).

EndoS also offers a route to improving the efficacy of therapeutic antibodies. EndoS can be used to generate deglycosylated antibodies, which exhibit immunosuppressive properties (95, 96). Serum IgG is present at concentrations of around 10 mg/ml and results in the almost-complete saturation of cellular Fc γ Rs. By stripping the receptors of endogenous IgG with EndoS, the receptor binding activity of mAbs can be dramatically enhanced if the mAb is introduced either after EndoS activity has been cleared from the serum or by the expression of the mAb as a glycoform resistant

to EndoS hydrolysis, such as oligomannose- or hybrid-type glycoforms (97).

Summary

A broad spectrum of opportunities for the medical exploitation of glycosylation has emerged. Importantly, several clinically effective therapeutics have already been developed. Intervening in cellular glycosylation pathways provides a route to the alleviation of many of the symptoms of congenital metabolic disorders. Some of these same drugs also have an impact on glycan-mediated virion assembly and offer an exciting prospect for the development of broad-spectrum antivirals against enveloped viruses. Further stages of the viral replicative cycle can be disrupted by considering their dependence on glycosylation, and this currently forms the basis of anti-influenza drugs and offers a route to the development of a new class of anti-inflammatory small-molecule drugs. The development of therapeutic glycoproteins has been greatly stimulated by the advances in recombinant cellular biosynthetic technologies that can produce defined glycoforms. mAbs with engineered glycosylation have emerged that display enhanced in vivo properties and have reached the clinic. Serum antibodies involved in autoimmunity can be inactivated by removal of their glycans by bacterial immune evasion factors, and this technology shows great promise in preclinical studies. Last, glycopeptides offer intriguing possibilities in the development of anticancer vaccines given their ability to stimulate both humoral and cellular immunity. In addition, the HIV glycan shield is proving to be an effective target for antibody neutralization. Design strategies that target conserved glycan clusters could contribute to the next generation of HIV vaccine candidates.

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Supplementary Materials
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The Bright Optical Flash and Afterglow from the Gamma-Ray Burst GRB 130427A

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The optical light generated simultaneously with x-rays and gamma rays during a gamma-ray burst (GRB) provides clues about the nature of the explosions that occur as massive stars collapse. We report on the bright optical flash and fading afterglow from powerful burst GRB 130427A. The optical and >100 -megaelectron volt (MeV) gamma-ray flux show a close correlation during the first 7000 seconds, which is best explained by reverse shock emission cogenerated in the relativistic burst ejecta as it collides with surrounding material. At later times, optical observations show the emergence of emission generated by a forward shock traversing the circumburst environment. The link between optical afterglow and >100 -MeV emission suggests that nearby early peaked afterglows will be the best candidates for studying gamma-ray emission at energies ranging from gigaelectron volts to teraelectron volts.

Gamma-ray bursts (GRBs) of long duration are associated with the collapse of massive stars to form black holes (1) or rapidly spinning, highly magnetized neutron stars (2). This collapse is believed to eject collimated relativistic jets that, through internal dissipation processes and collisions with the surroundings, generate luminous outbursts of electromagnetic radiation that have been detected at radio frequencies to very high (gigaelectron volt) gamma-ray energies. Most of the outburst energy is emitted in the gamma rays. But starting with the first observations establishing that GRBs occur at cosmological distances (3), correlative optical observations, in particular, have proven themselves as important tools for unraveling the nature of GRB explosions. Here, we present observations of the optical flash and early afterglow for a nearby burst that is bright enough in very-high-energy gamma rays to allow a detailed comparison of the >100 -MeV gamma-ray and optical light curves. These optical observations cover the critical early phases of the explosion, from the time interval before the event onset through the bright optical and prompt gamma-ray-emitting period and well into the early afterglow phase.

Starting on 27 April 2013 at 07:47:06.42 UTC (hereafter T_0), the Gamma Ray Burst Monitor (GBM) on the Fermi Satellite, the Burst Alert Telescope (BAT) on the Swift Satellite, and an armada of other space-based gamma-ray instru-

ments detected the onset of a powerful GRB (4, 5). This GRB, called GRB 130427A, had the largest gamma-ray fluence ($\sim 2.7 \times 10^{-3}$ erg/cm² in the 20- to 1200-keV band) measured in more than 18 years of operation by Konus-Wind (6) and set a record for duration of the >100 -MeV gamma-ray-emitting interval (5). Spectroscopy of the optical counterpart (7), coarsely localized by the Swift BAT and later refined by follow-up with optical telescopes, places the GRB at a redshift $z = 0.34$, a distance about five times closer than the typical GRB localized by Swift. However, even accounting for its proximity, the intense gamma-ray fluxes observed imply an apparent isotropic energy release of nearly 10^{54} ergs and rank 130427A among the more powerful GRBs ever detected (3). Subsequent optical monitoring

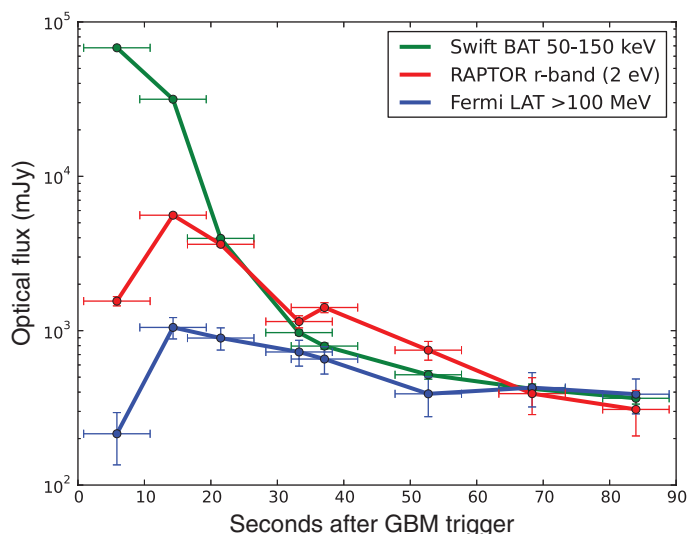
revealed the emergence of a broad-line supernova at the GRB location (8).

The Bright Optical Flash

This powerful GRB also generated an extremely bright flash of optical emission and a long-lived, bright optical afterglow. Three independent RAPTOR (Rapid Telescopes for Optical Response) full-sky monitoring telescopes (9), at locations in New Mexico and Hawaii, detected the emergence of a bright flash, temporally coincident with the onset of gamma-ray emission, at the location of GRB 130427A. The optical flash rapidly peaked at a magnitude of 7.03 ± 0.03 (unfiltered observations calibrated to Sloan r' band) in an exposure that covered the time interval from $T_0 + 9.31$ s to $T_0 + 19.31$ s. After the peak, the flash faded with a power-law flux decay with index $\alpha = -1.67 \pm 0.07$ ($\chi^2 = 0.68/5$ degrees of freedom) and was detected for ~ 80 s until it faded below the ~ 10 th magnitude sensitivity limit of the RAPTOR full-sky monitors.

The taxonomy for optical emission detected during the prompt gamma-ray emitting interval identifies two broad classes: prompt optical emission correlated with prompt gamma-ray emission (10–12) and early optical afterglow emission uncorrelated with the prompt gamma-ray emission (11, 13, 14). In context of the standard fireball model (15, 16), the prompt optical emission is attributed to internal shocks in an ultrarelativistic jet outflow generated by the central engine and the afterglow emission to external shocks generated by interaction with the surrounding medium. The prompt optical emission therefore reflects the impulsive energy injection into the jet, and the early afterglow emission measures the response of the jet-environment system to the energy injection. Bright optical flashes from reverse shocks were predicted on theoretical grounds (16, 17) before observational evidence was seen in GRB 990123

Fig. 1. A comparison of the relative flux variations measured for GRB 130427A by the Fermi LAT, RAPTOR, and the Swift BAT during the first 90 s after the GRB trigger. The >100 -MeV emission and the 50- to 150-keV emission have been integrated over the same time intervals as the optical exposures and multiplied by a scaling factor to allow comparison with the optical light curve. Both the optical and >100 -MeV light curves rise to a peak in the second interval, 50- to 150-keV emission peaks in the first interval. Horizontal error bars denote the time interval for the measurement; vertical error bars denote the 1σ uncertainty on the measured flux density.



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(13). The optical flash light curve for GRB 130427A shows a single peak delayed with respect to the kiloelectron volt–megaelectron volt (keV–MeV) prompt gamma-ray peak (Fig. 1) and a steep power-law flux that is consistent with the predictions of models for optical flashes from reverse shocks (17). Based on the above taxonomy, the brightness of the flash, and the rapid power-law flux

decay, it makes sense to associate the optical flash with reverse shock emission.

To explore the evolution of the broadband GRB spectrum during the optical flash, we used simultaneous measurements taken with the Fermi GBM and the Fermi Large Area Telescope (LAT) to construct spectral energy distributions (SEDs). Each snapshot of the time-evolving SED was formed

by integrating the GRB flux over the same time interval as the optical exposure. We found that the broadband SEDs (Fig. 2) varied rapidly during the first 40 s, and the optical measurements fell far from the values expected from extrapolation of the keV–MeV SED. However, as the intensity of the outburst declined during the next 40-s interval, the SED shape stabilized, and the optical measurements

Fig. 2. The spectral energy distributions measured for GRB 130427A during the early phases of the burst development. The points and solid lines represent actual measurements. The dotted straight lines indicate the extrapolation the keV–MeV measurements for comparison to the optical measurements. The dashed lines indicate the connection between energy bands and are not actual measurements. The measurements were obtained by RAPTOR, the Fermi GBM, and the Fermi LAT. The vertical errors bars (indicating the 1σ confidence interval) on the GBM measurements have been increased by 25% to allow for systematics, such as pulse pile-up, background subtraction during and after the repointing of Fermi, and the rapid spectral evolution during the exposure intervals. νF_ν is the energy flux measured at a given frequency. The horizontal error bars on the values at very high frequencies denote the frequency interval for the energy flux measurement.

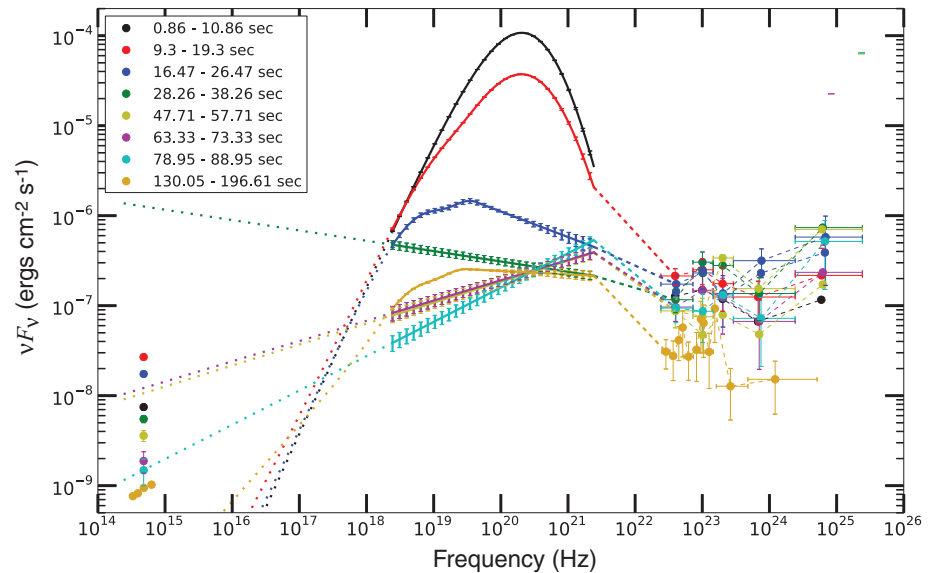


Fig. 3. Simultaneous multicolor measurements of the early optical afterglow from GRB 130427A. (Top) Light curves obtained using standard Sloan g' , r' , i' , and z' filters (24). The light curves show more structure than is captured by power-law fits, but the r' -band light curve can be characterized as a power-law decay with index ~ 0.7 before 270 s, ~ 1.1 between 270 and 3000 s, and 0.88 between 3000 and 7500 s. Also plotted for comparison, in blue, is the photon flux light curve for >100 -MeV emission measured by the Fermi LAT (4). **(Bottom)** The g' - r' color evolution of GRB 130427A. The red line indicates the mean g' - r' value (0.286 ± 0.018) measured before $T_0 + 1000$ s. After about $T_0 + 3000$ s, a bluer component emerges as the flux decay slows. Vertical error bars indicate the 1σ (or 68%) confidence interval on the measured quantity; horizontal error bars, when long enough to be visible, denote the measurement time interval.

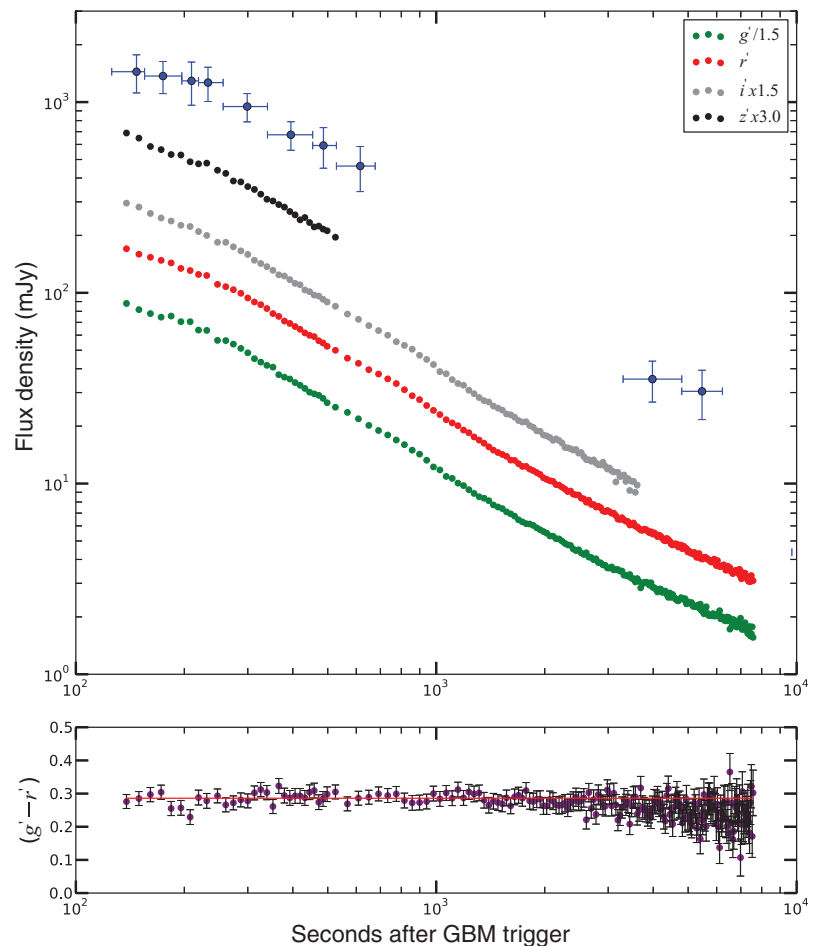
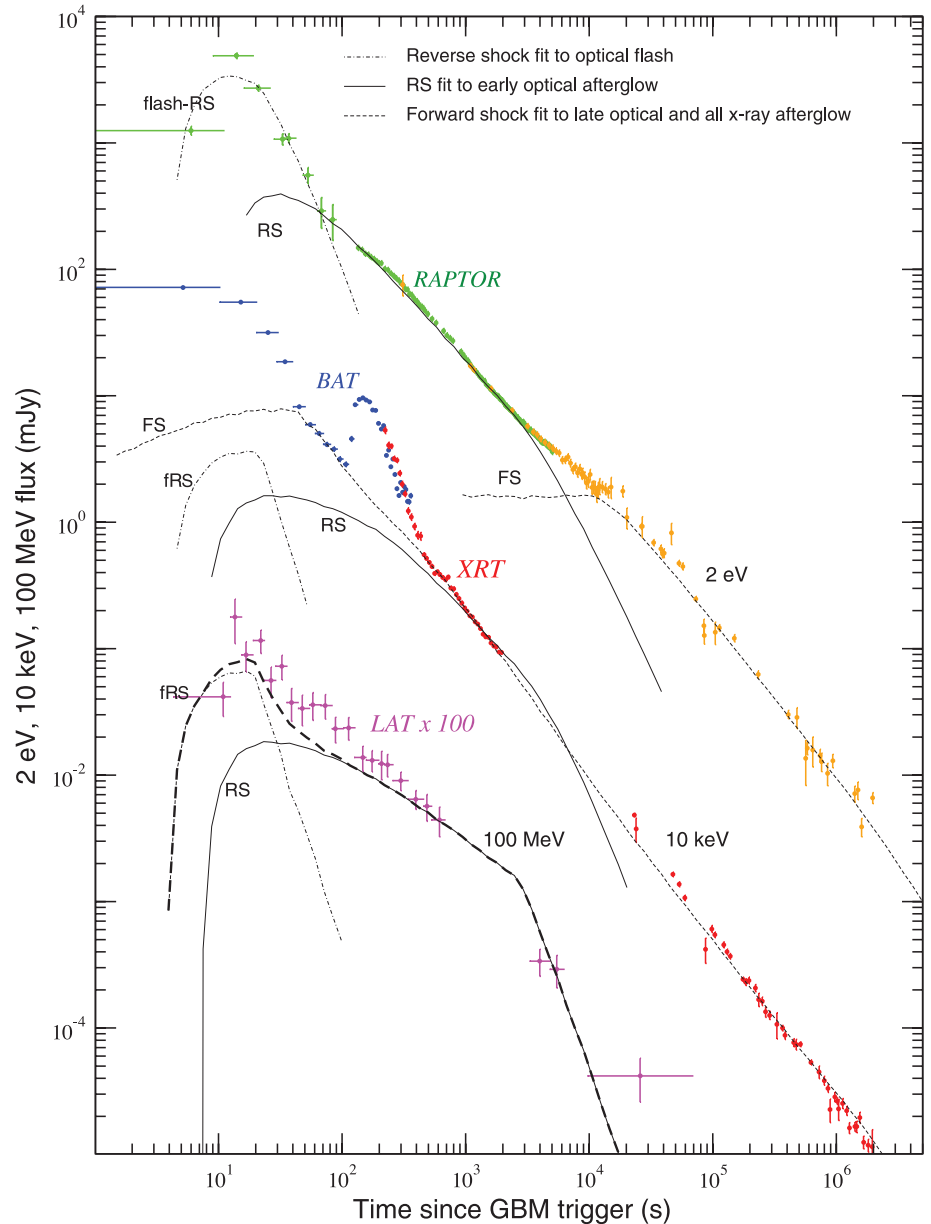


Fig. 4. Best-fit with a reverse forward shock model to the optical light curve of GRB afterglow 130427A. Three episodes of ejecta and energy injection are needed to explain the optical flash, the early optical emission (up to few kiloseconds), and the late optical emission (after a few kiloseconds). Here, each injection episode represents a change in the dynamical and microphysical parameters of the reverse shock in an otherwise continuous ejecta injection into the reverse shock. For the first two episodes, the optical emission arises from the reverse shock, and the kinetic energy of the incoming ejecta (6.10^{52} erg/sr and 4.10^{53} erg/sr, respectively) is less than that of the leading shock (10^{54} erg/sr). During the last injection episode, the optical afterglow emission arises from the forward-shock, with the incoming ejecta carrying 3.10^{54} erg/sr, which is more than that already existing in the forward shock (thus, its deceleration is mitigated by the energy injection). Other parameters are (i) first injection episode, flash reverse shock (fRS) onset at 4 s, end at 15 s, incoming ejecta Lorentz factor 730, magnetic field parameter 0.008, electron-energy parameter 0.006, index of electron power-law distribution with energy 1.9; (ii) second injection episode, reverse shock (RS, parameters in same order as above): 15 s, 3 ks, 1800, 0.0010, 0.012, 2.0; and (iii) third injection episode, forward shock (FS): 3 ks, >2 Ms, >100 , 3.10^{-4} , 0.14, 2.3, energy injection law $E \sim t^{0.3}$. The microphysical parameters for the forward shock are kept constant throughout the entire course of the event. We also require that the ambient medium have a windlike density stratification ($n \sim r^{-2}$, where n is the wind density in particles per unit volume, and r is the distance from the wind origin) corresponding to a GRB progenitor with a mass-loss rate-to-wind-speed ratio of 4.10^{-11} [units of (solar mass/year)/(km/s)]. Dot-dash lines show the model fits for the flash-phase emission, the solid lines show the reverse shock contributions during the early afterglow phase, and the dashed lines shows the contributions of the forward shock emission to the late optical afterglow and all phases of the x-ray afterglow. The reverse shock emission during the third injection episode is not shown and would be dimmer than that of the forward shock, if the reverse shock has the same microphysical parameters as the forward shock.



started to converge on the values predicted by a straightforward linear extrapolation of the keV-MeV SED. By the end of the optical flash, the optical-to-10-MeV spectrum is consistent with a single power law with index $\beta = -0.64$.

Sustained External Shocks and High-Energy Gamma Rays

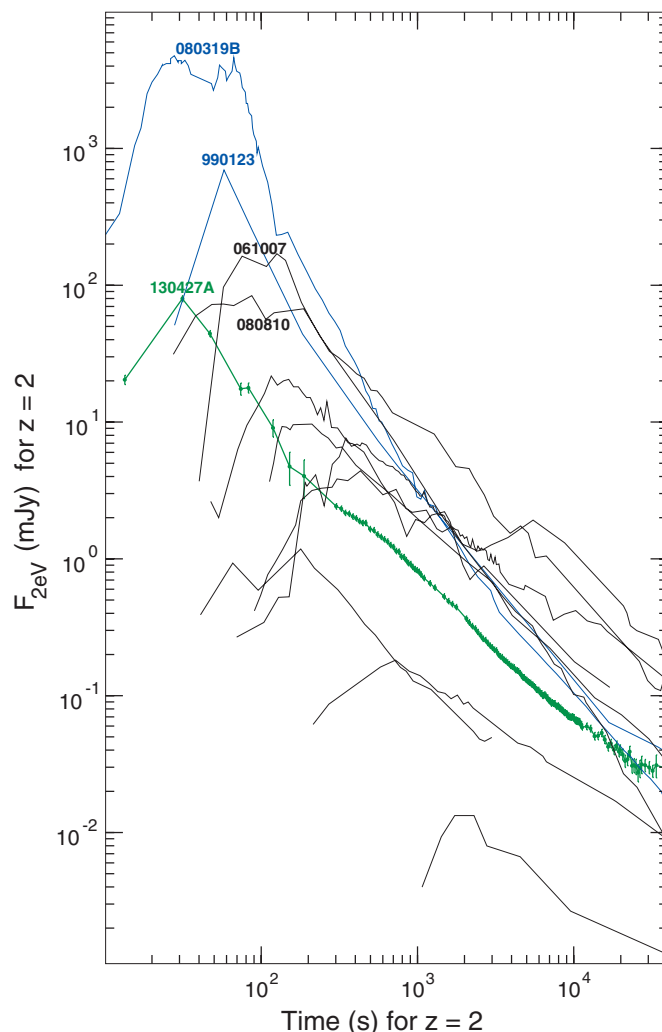
In response to the Swift BAT localization alert at 127.8 s after the GBM trigger, our RAPTOR response telescopes began unfiltered and simultaneous multicolor (g' , r' , i' , z') optical observations at $T_0 + 132.9$ s that continued until $T_0 + 7585.9$ s. This photometry begins near the peak of a prominent flare in keV-MeV x-rays and gamma rays that lasts until $\sim T_0 + 400$ s. The optical light curves show a smooth monotonic decline but no indication of the steep decline nor the break to a slower power-law decay at ~ 400 s measured at

x-ray energies (4). Instead, the structure of the optical light curve shows a steepening at about $T_0 + 270$ s. This steepening is essentially achromatic, and the color of the optical emission is consistent with a $\nu^{-0.70 \pm 0.05}$ spectrum and is constant until it starts to become bluer ($\nu^{-0.59 \pm 0.05}$) at ~ 300 s after the GBM trigger (see bottom panel of Fig. 3).

In marked contrast to the keV-MeV emission, the optical light curves after $T_0 + 100$ s show a strong similarity to the >100 -MeV photon flux light curve measured by the Fermi LAT (5). The LAT light curve has a break at ~ 300 s, just like the optical afterglow. Straightforward scaling of the RAPTOR optical light curve by a factor of $\sim 10^{-6}$ provides a reasonable description of the LAT observations out to $\sim T_0 + 7000$ s. This close correspondence argues for a common origin of both components in external shocks.

The optical light curve until $\sim T_0 + 3000$ s is best modeled by synchrotron emission from a reverse shock in a wind density profile. Most optical afterglows have been modeled with forward shocks in a homogeneous medium, but the peak brightness [~ 6 jansky (Jy)] and steep decay of the optical flash suggest origin in a reverse shock. The relative faintness of the radio afterglow peak (~ 1 mJy) also argues for generation by a reverse shock in a windlike medium (18). To explain the optical flash by reverse shock emission in a wind (R^{-2}) requires either a long-lived electron-energy injection up to ~ 40 s or a shorter one (~ 20 s), followed by adiabatic cooling. Figure 4 shows the best fit to the optical flash with the short interval of injection (on at $T_0 + 4$ s and off at $T_0 + 20$ s) and, for self-consistency, the same dynamical parameters that we infer from fits to the later afterglow forward shock emission discussed below.

Fig. 5. Comparison of the light curve from GRB 130427A (in green) with those measured for other prominent GRBs with peaked optical afterglows. All of the light curves have been transformed to show how they would appear if they had occurred at redshift $z = 2$. $F_{2\text{eV}}$, flux density at 2 eV.



This model employs an electron distribution with power-law energy index $p = 1.88$ and corresponds to an injected energy of 8.0×10^{53} ergs. The slower optical fading after the flash interval requires a second episode of energy injection to sustain the optical afterglow or a continuous outflow with a variable Lorentz factor (19). This sustained reverse shock model reproduces the closely tracking variability observed by the RAPTOR telescopes and the Fermi LAT and suggests that the optical emission, and most of the >100 -MeV emission, is generated by synchrotron emitting electrons that are accelerated by the reverse shocks.

By itself, this reverse shock model cannot explain the properties of the prompt keV-MeV emission, nor some of the properties of the late-time afterglows. The evolution to a bluer color after ~ 3000 s observed by RAPTOR and the slowing of the optical brightness decay suggest the emergence of a forward shock component. This transition to forward shock dominance at late times would also naturally explain the late-time x-ray light curve and the sustained >100 -MeV emission after 10,000 s. Emergence of a bluer optical component at late times is similar to the afterglow evolution of GRB 080319B—another burst

with a bright optical flash. For GRB 080319B, the color change was also interpreted as marking the transition from reverse shock emission dominance to forward shock dominance (12, 20, 21).

During most of the interval before $T_0 + 400$ s, the keV-MeV x-ray and gamma-ray emission is consistent with the standard assumption that the prompt emission is generated by internal shocks in the relativistic jet ejecta. Our predicted >100 -MeV flux from the reverse shock that generates the optical flash is slightly less (about a factor of 2) than the peak measured by the Fermi LAT (Fig. 4). But the keV-MeV flux is substantially underpredicted by at least a factor of 10, so the reverse shock in a wind model requires prompt emission to explain the keV-MeV emission and might require additional >100 -MeV emission to explain the LAT light-curve peak. In this picture, the keV-MeV light curve is a proxy that traces the injection of internal jet energy by the central engine. The keV-MeV emission therefore indicates two periods of substantial energy injection into the jet: the initial 20 s and a period from ~ 120 to 300 s. The interesting potential exception to prompt emission dominance in the keV-MeV range is the period just before onset

of the flare at $T_0 + 120$ s. During the interval $T_0 + 79$ s to $T_0 + 89$ s, the optical afterglow flux measured by RAPTOR falls right on the extrapolation of the power law (index ~ -0.6) measured by the GBM in the energy band from 10-keV to 20-MeV. This gamma-ray spectral slope is also similar to spectral slope that we measure for optical afterglow emission at later times. The notch in the keV-MeV light curve before the flare at $T_0 + 120$ may be providing a rare glimpse, similar to that seen in GRB 980923 (22), of afterglow emission at megaelectron volt energies between prompt emission intervals.

The exceptional optical properties observed for the optical flash and afterglow from GRB 130427A are mostly a result of burst proximity. The flash peak luminosity for GRB 130427A is among the most powerful events, but its value is consistent with the anticorrelation between peak time and peak luminosity (Fig. 5) found for optical afterglows (23). If optical afterglows and >100 -MeV gamma-ray afterglows have a common origin, then the optical afterglows that peak early should be the best candidates for detection at gigaelectron volt–teraelectron volt gamma-ray energies.

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Supplementary Materials

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Supplementary Text

Table S1

References (25–31)

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Fermi-LAT Observations of the Gamma-Ray Burst GRB 130427A

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The observations of the exceptionally bright gamma-ray burst (GRB) 130427A by the Large Area Telescope aboard the Fermi Gamma-ray Space Telescope provide constraints on the nature of these unique astrophysical sources. GRB 130427A had the largest fluence, highest-energy photon (95 GeV), longest γ -ray duration (20 hours), and one of the largest isotropic energy releases ever observed from a GRB. Temporal and spectral analyses of GRB 130427A challenge the widely accepted model that the nonthermal high-energy emission in the afterglow phase of GRBs is synchrotron emission radiated by electrons accelerated at an external shock.

Gamma-ray bursts (GRBs) are thought to originate from collapsing massive stars or merging compact objects (such as neutron stars or black holes), and are associated with the formation of black holes in distant galaxies. GRB 130427A was detected by both the Large Area Telescope (LAT) and the Gamma-ray Burst Monitor (GBM) aboard the Fermi Gamma-ray Space Telescope. The LAT is a pair-conversion telescope that observes photons from 20 MeV to >300 GeV with a 2.4-steradian field of view (*1*). The GBM consists of 12 sodium iodide (NaI, 8 keV to 1 MeV) detectors and two bismuth germanate (BGO, 200 keV to 40 MeV) detectors, positioned around the spacecraft to view the entire unocculted sky (*2*).

In the standard model of GRBs, the blast wave that produces the initial, bright prompt emission later collides with the external material surrounding the GRB (the circumburst medium) and cre-

ates shocks [see, e.g., (*3*)]. These external shocks accelerate charged particles, which produce photons through synchrotron radiation. Until this burst, the high-energy emission from LAT-detected GRBs had been well described by this model, but GRB 130427A challenges this widely accepted model. In particular, the maximum possible photon energy prescribed by this model is surpassed by the late-time high-energy photons detected by the LAT. The LAT detected high-energy γ -ray emission from this burst for almost a day, including a 95-GeV photon [which was emitted at 128 GeV in the rest frame at redshift $z = 0.34$ (*4*)] a few minutes after the burst began and a 32-GeV photon (43 GeV in the rest frame) after more than 9 hours. These were more energetic and were detected at considerably later times than the previous record holder, an 18-GeV photon detected by the Energetic Gamma Ray Experiment Telescope (EGRET) aboard the Compton

Gamma Ray Observatory more than 90 min after GRB 940217 began (*5*).

Observations

At 07:47:06.42 UTC on 27 April 2013 (T_0), while Fermi was in the regular survey mode, the GBM triggered on GRB 130427A. The burst was sufficiently hard and intense to initiate an autonomous repoint request (*6*)—a spacecraft slewing maneuver that keeps the burst within the LAT field of view for 2.5 hours, barring Earth occultation. At the time of the GBM trigger, the Swift Burst Alert Telescope (BAT) was slewing between two planned targets; the BAT triggered on the ongoing burst at 07:47:57.51 UTC immediately after completing the slew (*7*), 51.1 s after the GBM trigger. The CARMA millimeter-wave observatory localized this burst to R.A. = 173.1367°, Dec. = 27.6989° (J2000) with an uncertainty of 0.4 arc sec (*8*). The Rapid Telescopes for Optical Response (RAPTOR) detected bright optical emission from the GRB, peaking at a red-band magnitude of $R = 7.03 \pm 0.03$ around the GBM trigger time before fading to $R \approx 10$ about 80 s later (*9*). The Gemini-North observatory reported a redshift of $z = 0.34$ (*4*), and an underlying supernova has been detected (*10*). A total of 58 observatories have reported observations of this burst as of September 2013.

At the time of the GBM trigger, the GRB was 47.3° from the LAT boresight, well within the LAT field of view. The autonomous repoint request brought the burst to 20.1° from the LAT boresight, based on the position calculated by the GBM flight software. It remained in the LAT field of view for 715 s until it became occulted by Earth, reemerging from Earth occultation at $T_0 + 3135$ s. Within the first ~ 80 ks after the trigger, the LAT detected more than 500 photons with energies greater than 100 MeV associated with the GRB; the previous record holder was GRB 090902B, with ~ 200 photons (*11*). In addition, the LAT detected 15 photons with energies greater than 10 GeV (versus only 3 photons for GRB 090902B). Using the LAT Low Energy (LLE) event selection (*12*), which considerably increases the LAT effective collecting area to lower-energy γ -rays down to 10 MeV (with adequate energy reconstruction down to 30 MeV) (*13, 14*), thousands of counts above background were detected between T_0 and $T_0 + 100$ s.

Temporal Characteristics

The temporal profile of the emission from GRB 130427A varies strongly with energy from 10 keV to ~ 100 GeV (Figs. 1 and 2). The GBM light curves consist of an initial peak lasting for a few seconds, a much brighter multi-peaked emission episode lasting ~ 10 s, and a dim, broad peak at $T_0 + 120$ s, which fades to an undetectable level after ~ 300 s [also seen in the Swift light curve (*7*)].

The triggering pulse observed in the LLE (>10 MeV) light curve is more sharply peaked than the NaI- and BGO-detected emission at T_0 .

The LLE light curve between $T_0 + 4$ s and $T_0 + 12$ s exhibits a multi-peaked structure. Some of these peaks have counterparts in the GBM energy range, although the emission episodes are not perfectly correlated (e.g., the sharp spike in the LLE light curve at $T_0 + 9.5$ s is not relatively bright in the GBM light curves) because of the spectral evolution with energy.

The LAT-detected emission, however, does not appear to be temporally correlated with either the LLE or GBM emission beyond the initial spike at T_0 . Photons with energies greater than 1 GeV are first observed ~ 10 s after T_0 , after the brightest GBM emission has ended, consistent with a delayed onset of the high-energy emission (13). The delayed onset is not caused by a progressively increasing LAT acceptance due to slewing, because the slew started at $T_0 + 33$ s. Instead, it reflects the true evolution of the GRB emission.

GRB spectra are generally well described by phenomenological models such as the Band function (15) or the smoothly broken power law [SBPL (16)]. For the brightest LAT bursts, the onset of the GeV emission is delayed with respect to the keV-MeV emission and can be fit by an additional power-law component (13). This additional component usually becomes significant while the keV-MeV emission is still bright.

For GRB 130427A, however, the extra power-law component becomes significant only after

the GBM-detected emission has faded (Fig. 3). During the initial peak ($T_0 - 0.1$ s to $T_0 + 4.5$ s), there are only a few LAT-detected photons, and the emission is well fit by an SBPL. For the brightest part of the burst ($T_0 + 4.5$ s to $T_0 + 11.5$ s), we did not use the GBM-detected emission because of the substantial systematic effects caused by extremely high flux (17); however, there are no photons with energies greater than 1 GeV in this time interval, and the energy spectrum above 30 MeV is well described by a single power law without a break (Fig. 3) (note that the LAT did not suffer from any pile-up issues). Photons with energies greater than 1 GeV are detected in the last time interval ($T_0 + 11.5$ s to $T_0 + 33.0$ s), including a 73-GeV photon at $T_0 + 19$ s. Unlike other bright LAT bursts, the LAT-detected emission from GRB 130427A appears to be temporally distinct from the GBM-detected emission, which suggests that the GeV and keV-MeV photons arise from different emission regions or mechanisms.

Temporally Extended High-Energy Emission

To characterize the temporally extended high-energy emission, we performed an unbinned maximum likelihood analysis of the LAT data for $E > 100$ MeV. We modeled the LAT photon spectrum as a power law with a spectral index α [i.e., the spectrum $N(E) \propto E^\alpha$]. We found evidence of spectral evolution during the high-

energy emission. In contrast to another study (18) that used longer time intervals in the spectral fits, we found that the LAT ($E > 100$ MeV) spectrum of the GRB is well described by a power law at all times, but with a varying spectral index (17).

During the first pulse around T_0 , emission at >100 MeV is faint and soft; the pulse contains only a few photons, and their energies are all <1 GeV (Fig. 2). This is followed by a period during which there is no significant emission at >100 MeV, while the GBM emission is at its brightest. Starting around $T_0 + 5$ s, emission at >100 MeV is detectable again but remains dim until $\sim T_0 + 12$ s. The spectral index fluctuates between $\alpha \sim -2.5$ and $\alpha \sim -1.7$. At late times (longer than $T_0 + 300$ s), we measured typical spectral indices of $\alpha \sim -2$, consistent with the indices of other LAT bursts (13). During the time intervals with the hardest spectra, the LAT observed the highest-energy photons—such as the 73-GeV photon at $T_0 + 19$ s and the record-breaking 95-GeV photon at $T_0 + 244$ s—that severely restrict the possible mechanisms that could generate the high-energy afterglow emission (table S2).

The temporally extended photon flux light curve is better fit by a broken power law than by a single power law. We found a break after a few hundred seconds, with the temporal index steepening from -0.85 ± 0.08 to -1.35 ± 0.08

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($\chi^2/\text{df} = 36/19$ for a single power law, 16/17 for a broken power law). In contrast, a break is not statistically preferred in the energy flux light curve ($\chi^2/\text{df} = 14/18$ for a single power law, 13/17 for a broken power law), probably because of the larger statistical uncertainties. For a single power-law fit to the energy flux light curve, we found a temporal index of -1.17 ± 0.06 , consistent with other LAT bursts (13).

The GBM and Swift energy flux light curves are also shown in Fig. 2. The Swift X-Ray Telescope (XRT) began observing the burst at $T_0 + 190$ s; the reported XRT + BAT (0.3 to 10 keV) light curve is a combination of XRT data and BAT-detected emission (15 to 150 keV) extrapolated down into the energy range of the XRT. The XRT + BAT light curve shows the unabsorbed flux in the range 0.3 to 10 keV (7). During the initial part of the burst, the GBM (10 keV to 10 MeV) light curve peaks earlier than both the XRT + BAT (0.3 to 10 keV) and LAT (>100 MeV)

light curves. The GBM light curve peaks again at $\sim T_0 + 120$ s [see also (7)], whereas the LAT light curve shows a sharp and hard peak at $T_0 + 200$ s. The BAT + XRT light curve peaks again as well at the same time as the LAT light curve, but the peak is much broader.

Interpretation

The energetics of GRB 130427A place it among the brightest LAT bursts. For GRB 130427A, the fluence at 10 keV to 20 MeV measured with the GBM in the 400 s following T_0 is $\sim 4.2 \times 10^{-3}$ ergs cm^{-2} . The issue with pulse pile-up and the uncertainties in the calibration of the GBM detectors contribute to a systematic error that we estimate to be less than 20%; the statistical uncertainty [0.01×10^{-3} ergs cm^{-2}] is negligible with respect to the systematic one (17). The fluence at >100 MeV measured with the LAT in the 100 ks following T_0 is $7 (\pm 1) \times 10^{-4}$ ergs cm^{-2} . The total LAT fluence is therefore $\sim 20\%$ of the GBM flu-

ence, similar to other bright LAT GRBs (13, 19). For a total fluence at 10 keV to 100 GeV of 4.9×10^{-3} ergs cm^{-2} , the total apparent isotropic γ -ray energy (i.e., the total energy release if there were no beaming) is $E_{\gamma, \text{iso}} = 1.40 \times 10^{54}$ ergs, using a flat Λ CDM cosmology with reduced Hubble constant $h = 0.71$ and dark energy density $\Omega_\Lambda = 0.73$; this value implies a luminosity distance of 1.8 Gpc for $z = 0.34$. This value of $E_{\gamma, \text{iso}}$ is only slightly less than the values for other bright LAT hyperenergetic events, which include GRB 080916C, GRB 090902B, and GRB 090926A (19).

The emission region must be transparent against absorption by photon-photon pair production, which has a significant effect at the energies of the LAT-detected emission. Viable models of GRBs therefore require highly relativistic, jetted plasma outflows with bulk jet Lorentz factors Γ greater than ~ 100 (20). The 73-GeV photon at $T_0 + 19$ s (table S2) provides the most stringent limit on Γ . Assuming that the variability time scale re-

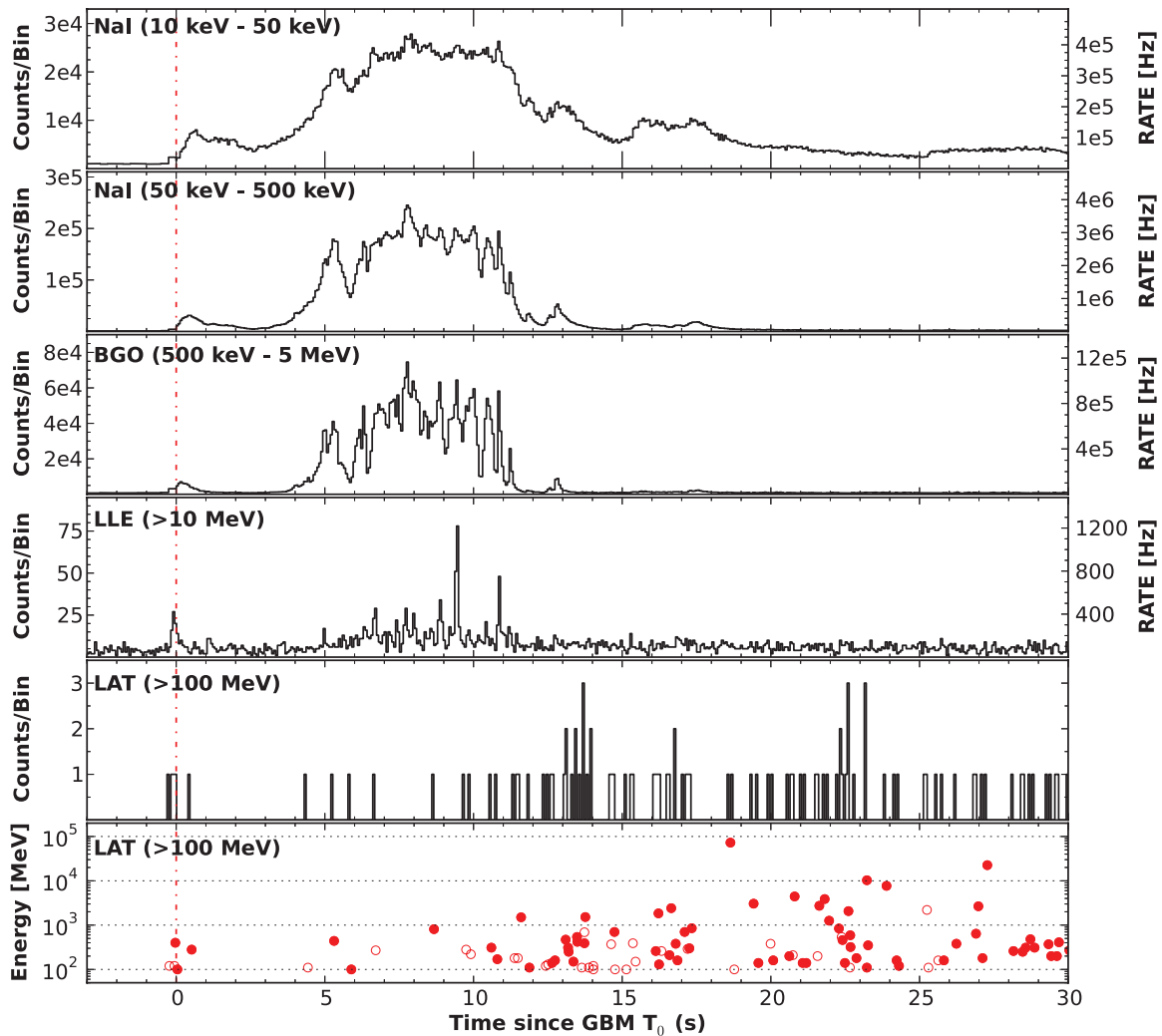


Fig. 1. Light curves for the Fermi-GBM and LAT detectors during the brightest part of the emission in 0.064-s bins, divided into five energy ranges. The Nal and BGO light curves were created from a type of GBM data (continuous time or CTIME) that does not suffer from saturation effects induced by the extreme brightness of

this GRB (17); for these light curves, we used Nal detectors 6, 9, and 10, and BGO detector 1. The open circles in the bottom panel represent the individual LAT “transient” class photons and their energies; the solid circles indicate photons with a >0.9 probability of being associated with this burst (17).

flects the size of the emitting region, and that the MeV and GeV emissions around the time of the 73-GeV photon at $T_0 + 19$ s are cospatial, the requirement that the optical depth due to $\gamma\gamma$ opacity be less than 1 then implies that the minimum bulk Lorentz factor is $\Gamma_{\min} = 455^{+16}_{-13}$. Here, a SBPL fit to the GBM spectrum in the interval 11.5 to 33.0 s (table S1) and a minimum variability time scale of 0.04 ± 0.01 s are used (17). The cospatial as-

sumption is, however, questionable given the different time histories in the MeV and GeV emission. Moreover, values of $\Gamma_{\min}$ that are smaller by a factor of 2 to 3 can be realized for models with time-dependent γ -ray opacity in a thin-shell model (21).

The delayed onset of the LAT-detected emission with respect to the GBM-detected emission is an important clue to the nature of GRBs (13). For

GRB 130427A, the LAT-detected emission becomes harder and more intense after the GBM-detected emission has faded (Fig. 3). This suggests that the GeV emission is produced later than the keV-MeV emission and in a different region. In particular, if the keV-MeV emission comes from interactions within the outflow itself, the GeV emission arises from the outflow's interactions with the circumburst medium.

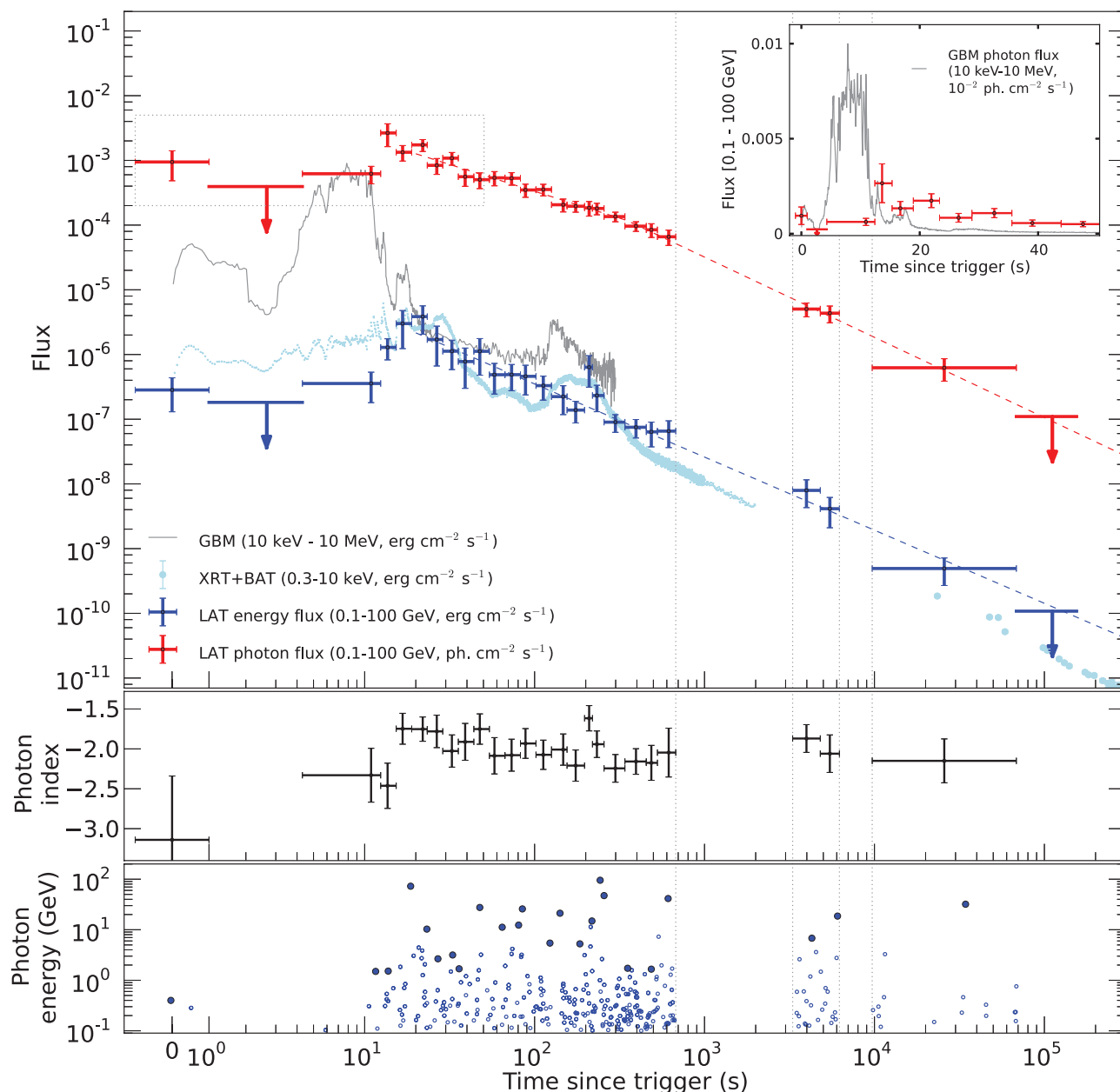


Fig. 2. Temporally extended LAT emission. Top: LAT energy flux (blue) and photon flux (red) light curves. The photon flux light curve shows a significant break at a few hundred seconds (red dashed line), whereas the energy flux light curve is well described by a single power law (blue dashed line). The 10 keV to 10 MeV (GBM, gray) and 0.3 to 10 keV (XRT + BAT, light blue) energy flux light curves are overplotted. The inset shows an expanded view of the first 50 s with a linear axes, with the photon flux light curve from the GBM (in units of 10^{-2} photons $\text{cm}^{-2} \text{s}^{-1}$) plotted in gray for comparison. Middle: LAT photon index. Bottom: Energies of all the photons with prob-

abilities $>90\%$ of being associated with the GRB (17). Solid circles correspond to the photon with the highest energy for each time interval. Note that the photons plotted here are “source” class photons, whereas the photons in Figs. 1 and 3 are “transient” class photons (17). The vertical gray lines indicate the first two time intervals during which the burst was occulted by Earth. As the autonomous repoint request moved the center of the LAT field of view toward the GRB position, the effective collecting area in that direction increased, so that after ~ 100 s the rate of photons increased even though the intrinsic flux decreased.

The explosive relativistic outflow of a GRB sweeps up and drives a shock into the circumburst medium. The medium could have, for instance, a uniform density n_0 (cm^{-3}) or a $n(r) \propto r^{-2}$ density profile resulting from the stellar wind of the type Ic supernova progenitor star associated with GRB 130427A (10). The LAT observations of GRB 130427A challenge the scenario in which the GeV photons are nonthermal synchrotron radiation emitted by electrons accelerated at the external forward shock (22, 23). In this model, the time of the brightest emission corresponds to the time t_{dec} when most of the outflow energy is transferred to the shocked external medium. The Lorentz factor $\Gamma(t_{\text{dec}})$ of the shock outflow in the external medium at the deceleration time t_{dec} is a lower limit for the initial bulk outflow Lorentz factor Γ_0 , because a relativistic reverse shock would lower the shocked fluid Lorentz factor below Γ_0 , and the engine time scale T_{GRB} can be longer than t_d , the deceleration time scale for an impulsive explosion. The activity of the central engine that produces the blast wave is revealed by the keV-MeV emission from particles accelerated at colliding-wind shocks. For GRB 130427A, the GeV emission starts to decay as a power law in time by $t \approx 20$ s (Fig. 2), and most of the keV-MeV radiation has subsided by $t \approx 12$ s (Fig. 1). The blast wave is in the self-similar deceleration phase at $t > t_{\text{dec}} = \max[t_d, T_{\text{GRB}}]$,

where T_{GRB} is the engine time scale (over which most of the outflow energy was released). Here, $t_d(\text{s}) \approx 2.4[(E_{\gamma, \text{iso}}/10^{54} \text{ erg})/n_0]^{1/3}/(\Gamma_0/1000)^{8/3}$ for a uniform external medium, and $t_d(\text{s}) \approx 6.3(E_{\gamma, \text{iso}}/10^{55} \text{ erg})(0.1/A_*)(500/\Gamma_0)^4$ for a stellar wind medium of density $\rho = AR^{-2}$, with $A^* = A/5 \times 10^{11} \text{ g cm}^{-1}$.

Most of the fluence from GRB 130427A was radiated before $t \approx 12$ s, which suggests that $t_d \lesssim 12$ to 15 s. Defining $t_1 = t_d/(10 \text{ s})$ yields $t_1 \approx 1$ to 2, which gives $\Gamma(t_{\text{dec}}) \approx 540[E_{55}/t_1^3 n_0(\text{cm}^{-3})]^{1/8}$ for the uniform-density case, where $E_{55} = E_{\gamma, \text{iso}}/(10^{55} \text{ erg})$ is the isotropic energy release of the GRB. For a wind medium, $\Gamma(t_{\text{dec}}) \approx 450\{E_{55}/[(A^*/0.1)t_1]\}^{1/4}$. Both values are close to the $\gamma\gamma$ opacity estimate of Γ_{min} .

The presence of high-energy photons at times $t \gg t_{\text{dec}}$ (table S2) is incompatible with these γ rays having a synchrotron origin. Equating the electron energy loss time scale due to synchrotron radiation with the Larmor time scale for an electron to execute a gyration gives a conservative limit on the maximum synchrotron photon energy $E_{\text{max, syn}} \approx 2^{3/2}[27/(16\pi\alpha_f)]m_e c^2 \Gamma(t)/(1+z) \approx 79[\Gamma(t)] \text{ MeV}$, where α_f is the fine-structure constant (17). Using $\Gamma(t)$ derived by Blandford and McKee (24) in the adiabatic limit, we find that the maximum synchrotron photon energy $E_{\text{max, syn}} \ll 7(E_{55}/n_0)^{1/8}[t/200 \text{ s}]^{-3/8} \text{ GeV}$, which agrees with results from integration over surfaces of equal arrival time in the self-similar regime

(25), when a scaling factor of $27/16\pi$ is included (17). The presence of a 95-GeV photon at $T_0 + 244$ s (Fig. 4 and table S2) is incompatible with a synchrotron origin even for conservative assumptions about Fermi acceleration. This conclusion holds for adiabatic and radiative external shocks in both uniform or wind media [see also (26)]. The question of a wind or uniform-density model is not settled, but combined forward and reverse shock blast-wave model fits to the radio through x-ray emission from 0.67 days to 9.7 days after the GRB favor a wind medium (27), whereas inferences from Swift and LAT data suggest a uniform environment around GRB 130427A (7). Even in the extreme case where acceleration is assumed to operate on a time scale shorter than the Larmor time scale by a factor of 2π , synchrotron radiation cannot account for the presence of high-energy radiation in the afterglow. Synchrotron emission above ~ 100 GeV is still possible, however, if an acceleration mechanism faster than the Fermi process is acting, such as magnetic reconnection [e.g., (28)].

The 95-GeV photon in the early afterglow and the 32-GeV photon at $T_0 + 34.4$ ks therefore cannot originate from lepton synchrotron radiation in the standard afterglow model with shock Fermi acceleration (Fig. 4). If the emission mechanism for the GeV photons is not synchrotron radiation, the highest-energy photons can still be

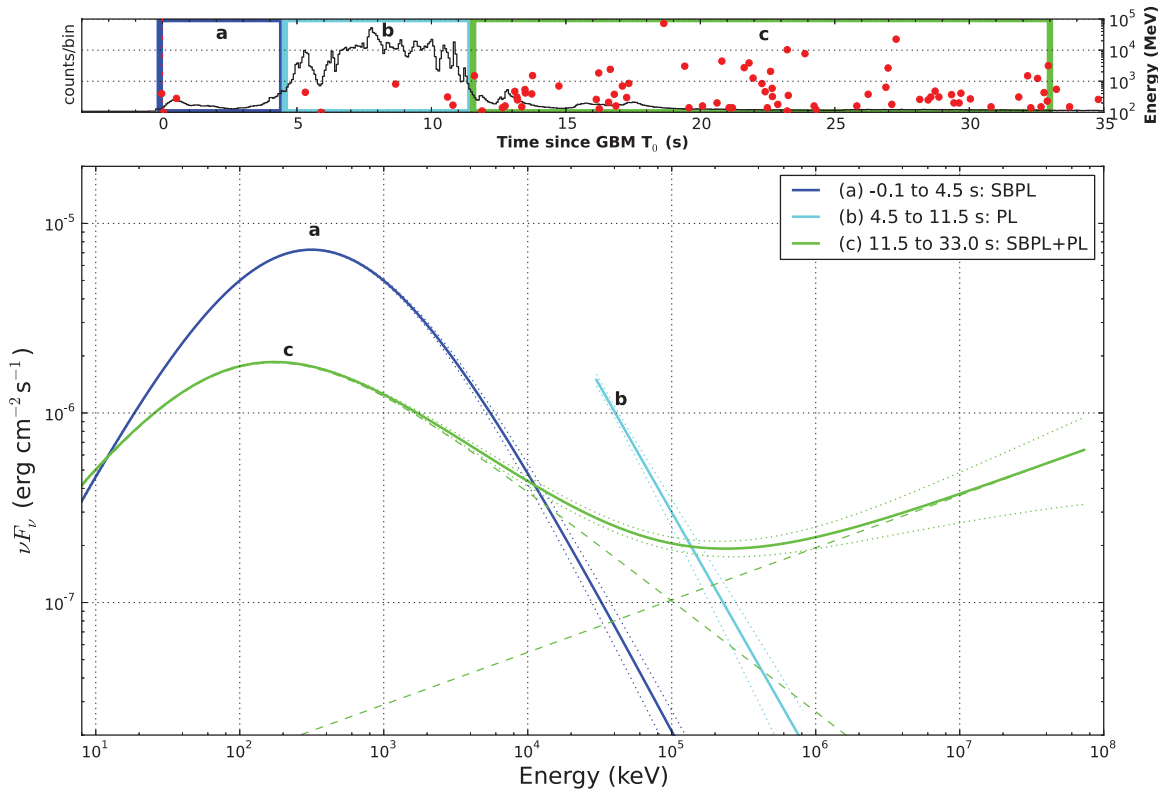


Fig. 3. Time-resolved spectral models for the GBM- and LAT-detected emission. Top: The combined NaI and BGO light curve from Fig. 1 (arbitrarily scaled) with the LAT-detected photons overplotted (same as the solid circles in Fig. 1). The time intervals are colored to correspond with the spectral models in the lower plot. The GBM data between 4.5 and 11.5 s are not included

because they are substantially affected by pulse pile-up (17). Bottom: The models (thick lines) that best fit the data are plotted with 1σ error contours (thin dashed lines). Each curve ends at the energy of the highest-energy LAT photon detected within that time interval. An extra power law is statistically significant when fitting the data from $T_0 + 11.5$ s to $T_0 + 33.0$ s (17).

produced by lepton Compton processes. Synchrotron self-Compton (SSC) γ -rays, made when target synchrotron photons are Compton-scattered by the same jet electrons that emit the synchrotron emission, are unavoidable. SSC emission is expected to peak at TeV and higher energies during the prompt phase (although no GRB has been detected at TeV energies) and would cause the GeV light curve to flatten and the LAT spectrum to harden when the peak of the SSC component passes through the LAT waveband (29–31). Such a feature may be seen in the light curves of GRB 090902B and GRB 090926A at 15 to 30 s after T_0 (13), but no such hardening or plateau associated with the SSC component is observed in the LAT light curve of GRB 130427A, although extreme parameters might still allow an SSC interpretation [see, e.g., (32)]. Except for the hard flare at $t \approx 250$ s and a possible softening at ~ 3000 s [and therefore not associated with a probable beaming break at $t \approx 0.8$ days (7)], neither the integral photon nor energy-flux light curves in Fig. 2 show much structure or strong evidence for temporal or spectral variability from $t = 20$ s to $t = 1$ day. The NuSTAR observations (33) of the late-time hard x-ray afterglow also suggest that a single spectral component produces the emission from optical to multi-GeV energies. If this emission is indeed synchrotron radiation, then the standard afterglow shock model must be modified to account for the highest-energy photons detected by the LAT.

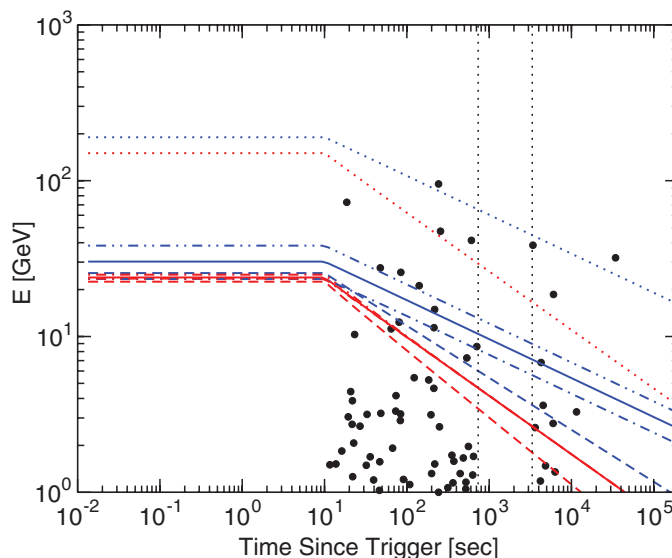
These considerations suggest that other extreme high-energy radiation mechanisms may be operative, such as external Compton processes. The most intense source of target photons is the

powerful engine emissions, as revealed by the GBM and XRT prompt emission. A cocoon or remnant shell is also a possible source of soft photons, but unless the target photon source is extended and radiant, it would be difficult to model the nearly structureless LAT light curve over a long period of time. Given the similarity between the XRT and LAT light curves (Fig. 2), afterglow synchrotron radiation made by electrons accelerated at an external shock would also be the favored explanation for the LAT emission, but this is inconsistent with the detection of high-energy photons at late times.

The photon index of GRB 130427A, ~ -2 , is similar to those found in calculations of electromagnetic cascades created when the γ -ray opacity of ultrahigh energy (UHE, >100 TeV) photons in the jet plasma is large (34). An electromagnetic cascade induced by ultrarelativistic hadrons would be confirmed by coincident detection of neutrinos, but even for GRB 130427A with its extraordinary fluence, only a marginal detection of neutrinos is expected with IceCube, and none has been reported (35). Because the UHE γ -ray photons induce cascades both inside the radiating plasma and when they travel through intergalactic space [e.g., (36, 37)], a leptonic or hadronic cascade component in GRBs, which is a natural extension of colliding shell and blast wave models, might be required to explain the high-energy emission of GRB 130427A provided that the required energies are not excessive. The observations described above demonstrate nonsynchrotron emission in the afterglow phase of the bright GRB 130427A, contrary to the hitherto standard model of GRB afterglows.

Fig. 4. Curves of maximum synchrotron photon energy.

The black dots show the LAT detection times of photons with energies greater than 1 GeV and $>90\%$ probability of association with GRB 130427A. Adiabatic and radiative predictions for maximum synchrotron photon energy in uniform interstellar medium (ISM) and wind environments are plotted using the relations described in (17). Red and blue curves refer to the ISM and wind cases, respectively. The solid and dashed lines refer to the adiabatic and radiative cases with $\Gamma_0 = 1000$, and the dot-dashed and double dot-dashed lines represent the adiabatic case with $\Gamma_0 = 500$ and $\Gamma_0 = 2000$, respectively. The dotted lines show an extreme possibility where acceleration takes place on the inverse of the Larmor angular frequency, in the case of an adiabatic blast wave with $\Gamma_0 = 1000$. For cases with uniform external medium, $E_{\text{iso}}(10^{55} \text{ erg})/n_0(\text{cm}^{-3}) = 1$. The wind normalization was chosen to give the same value of t_4 for both wind and ISM cases. The vertical dotted lines show periods of Earth avoidance when the LAT could not observe GRB 130427A.



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Supplementary Materials

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GRB 130427A: A Nearby Ordinary Monster

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Long-duration gamma-ray bursts (GRBs) are an extremely rare outcome of the collapse of massive stars and are typically found in the distant universe. Because of its intrinsic luminosity ($L \sim 3 \times 10^{53}$ ergs per second) and its relative proximity ($z = 0.34$), GRB 130427A reached the highest fluence observed in the γ -ray band. Here, we present a comprehensive multiwavelength view of GRB 130427A with Swift, the 2-meter Liverpool and Faulkes telescopes, and by other ground-based facilities, highlighting the evolution of the burst emission from the prompt to the afterglow phase. The properties of GRB 130427A are similar to those of the most luminous, high-redshift GRBs, suggesting that a common central engine is responsible for producing GRBs in both the contemporary and the early universe and over the full range of GRB isotropic energies.

Gamma-ray burst (GRB) 130427A was the brightest burst detected with Swift (1), as well as with several γ -ray detectors on-board other space missions. It was also the brightest and longest burst detected above 100 MeV, with the most energetic photon detected at 95 GeV (2). It was detected with Fermi–Gamma-ray Burst Monitor (GBM) (3) at T_0 , GBM = 47:06:42 UT on 27 April 2013. Hereafter, this time will be our reference time T_0 . The Burst Alert Telescope (BAT) (4) on-board Swift triggered on GRB 130427A at time (t) = 51.1 s, when Swift completed a preplanned slew. The Swift slew to the source started at $t = 148$ s and ended at $t = 192$ s. The Swift UltraViolet Optical Telescope (UVOT) (5) began observations at $t = 181$ s, whereas observations with the Swift X-ray Telescope (XRT) (6) started at $t = 195$ s (7). The structure of the γ -ray light curve revealed by the Swift-BAT in the 15- to 350-keV band (Fig. 1) can be divided in three main episodes: an initial peak, beginning at $t = 0.1$ s and peaking at $t = 0.5$ s; a second large peak, showing a complex structure with a duration of ~ 20 s; and a third, much weaker episode starting at $t \sim 120$ s, showing a fast rise/exponential decay behavior. The overall duration of the prompt emission was T_{90} (15 to 150 keV) = 276 ± 5 s (the time containing 90% of the fluence) calculated over the first 1830 s of BAT observation from T_0 , GBM. During the early phases of the γ -ray emission, strong spectral variability was observed (Fig. 1). A marked spectral hardening was observed during the prompt main event. With a total fluence $F = (4.985 \pm 0.002) \times 10^{-4}$ erg cm⁻² in the 15- to 150-keV band, GRB 130427A reached the highest fluence ob-

served for a GRB by Swift. The 0.02- to 10-MeV fluence measured by Konus-Wind (8) for the main emission episode (0 to 18.7 s) is $(2.68 \pm 0.01) \times 10^{-3}$ erg cm⁻², with a spectrum peaking at $E_{\text{peak}} = 1028 \pm 8$ keV, whereas the fluence of the emission episode at (120 to 250 s) is $\sim 9 \times 10^{-5}$ erg cm⁻², with a spectrum peaking at ~ 240 keV (9).

This event was extremely bright also in the optical and it was immediately detected with various robotic telescopes. In particular, the Raptor robotic telescope detected a bright optical counterpart already at $t = 0.5$ s (10). Optical spectroscopy of the afterglow determined the redshift to be $z = 0.34$ (11); a UVOT UV grism spectrum (7) was also acquired. At this distance, the rest frame 1-keV to 10-MeV isotropic energy is $E_{\text{iso}} = 8.1 \times 10^{53}$ erg, and the peak luminosity is $L_{\text{iso}} = 2.7 \times 10^{53}$ erg s⁻¹. According to the luminosity function of Salvaterra *et al.* (12), we expect one event like GRB 130427A every > 60 years. In the nearby universe ($z \lesssim 0.4$, corresponding to an age of ~ 10 billion years), only a handful of long GRBs have been detected. These GRBs are usually characterized by a low overall isotropic energy ($E_{\text{iso}} \leq 10^{52}$ erg) and are associated with supernovae (SNe) types Ib and Ic, characterized by broad spectral lines indicating high expansion velocities, called hypemovae (13). GRB 130427A is instead a powerful GRB, such as the ones typically detected at much higher redshifts ($z > 1$, with a mean $z \sim 2$ corresponding to an age of ~ 3 billion years). The detection of a nearby and extremely powerful GRB gives us the opportunity to test, on the one hand, whether this GRB has the same properties of the cosmological GRBs and, on the

other hand, whether also such bright GRBs are associated with SNe. Up to now, SNe have been associated only to under (or mildly) energetic GRBs in the local universe. Because a supernova associated with this burst, SN 2013cq, has been detected (14), we are now sure that SNe are also associated with very powerful GRBs, not only to low power bursts [fig. S6 and figure 1 of (14)]. Naive energetic arguments might suggest that in powerful GRBs, there is not enough power left for a strong SN; GRB 130427A definitively proves that this is not the case.

The overall behavior of the x-ray afterglow light curve has been characterized with the main contribution of the XRT on-board Swift and two additional relevant detections from the Monitor of All-sky X-ray Image (MAXI) experiment (15) in the gap between the first and the second Swift-XRT observations (Fig. 2). The early light curve, starting from $t = 260$ s, is characterized by an initially steep decay with a slope $\alpha_{0,x} = 3.32 \pm 0.17$, which is consistent with high-latitude emission (16, 17); a break at $t_{1,x} = 424 \pm 8$ s; and

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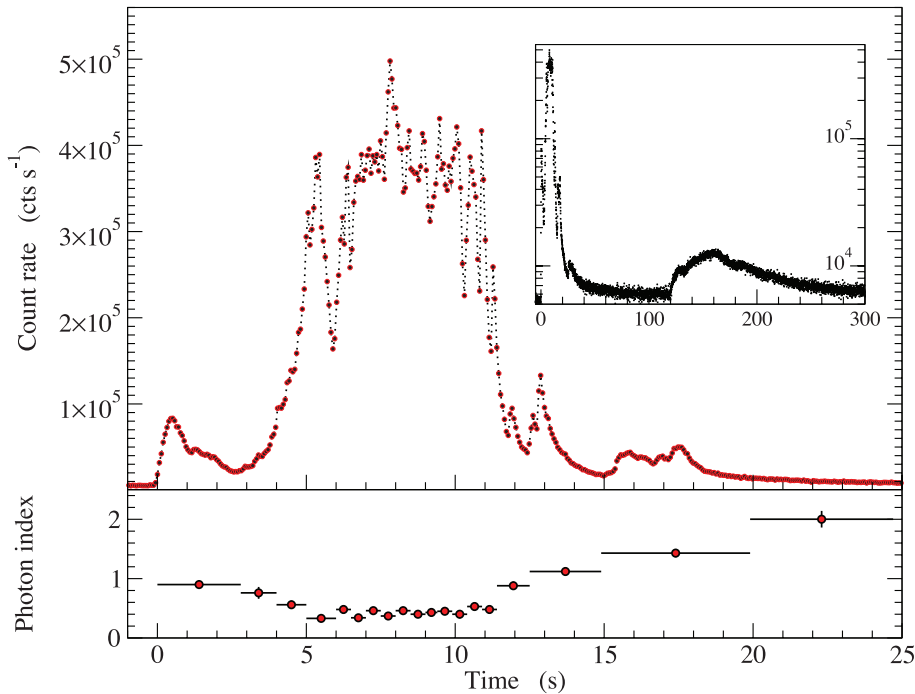


Fig. 1. Details of the Swift BAT light curve in the 15- to 350-keV band. (Top) The BAT light curve with a binning time of 64 ms. (Inset) The BAT light curve up to 300 s, plotted on a log intensity scale, showing a fast rise/exponential decay feature starting at $t \sim 120$ s. (Bottom) The photon index values of a power-law model fit to the BAT spectrum in the 15 to 150 keV energy range.

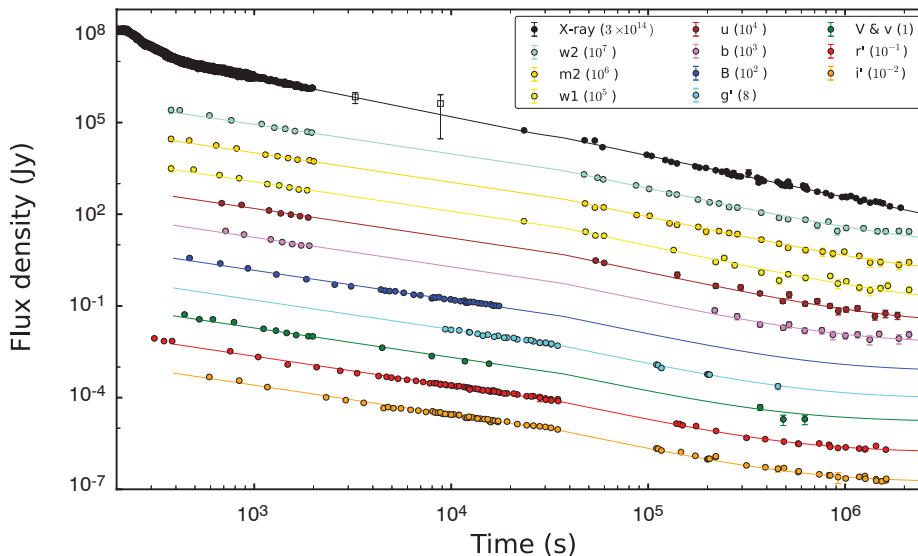


Fig. 2. Light curves for GRB 130427A in different wavebands. Swift UV and visible filters ($w2$, $m2$, $w1$, u , b , v); B , V , r' , and i' filters correspond to Faulkes Telescope North; r' , and i' to the Liverpool Telescope; g' , r' , and i' to the MITSuME telescopes (7). The scaling factors for the flux density in different filters is shown in the inset; the scaling factor for the x-ray light curve (flux integrated in the 0.3 to 10 keV range) (7) is also shown. The x-ray light curve also includes two MAXI data points at $t = 3257$ s and $t = 8821$ s (empty squares). The fit performed over all the light curves required 24 free parameters (curve normalizations, host galaxy optical flux in each band, three temporal slopes, and two breaks). Because of short-term low-level variability superposed to the long-term behavior and possibly residual inhomogeneity of optical data taken from different telescopes, we added in quadrature a 9% systematic error in the optical and 5% in the x-rays. Final fit yielded $\chi^2 = 543.02/565$ degrees of freedom. A contribution from the host galaxy has been taken into account in the optical bands by fitting a constant flux of ~ 0.01 millijansky for the reddest bands, corresponding to $r_{\text{HG}} = 21.26$ mag as tabulated in the Sloan Digital Sky Survey catalog. As a test of consistency, we performed the fit using a broken power law for the B , g' , r' , and i' filters individually. Uncertainties are larger, but we do find that the values of t_{break} , as well as the decay indices before and after the break time, are still consistent, within the errors, with the values obtained by the overall fit.

followed by a flatter decay with index $\alpha_{1,x} = 1.28 \pm 0.01$. A further break at $t_{2,x} = 48 \pm 22$ ks is statistically needed (3.8σ) to account for a further steepening to $\alpha_{2,x} = 1.35 \pm 0.02$ (all errors are derived for $\Delta\chi^2 = 2.7$).

The light curves in the optical and UV derived from the UVOT, as well as from ground-based telescopes (Liverpool telescope, Faulkes Telescope North, and MITSuME Telescopes) are shown in Fig. 2. All optical light curves are well fitted by a broken power law with $\alpha_1 = 0.96 \pm 0.01$, $t_{\text{break}} = 37.4^{+4.7}_{-4.0}$ ks, and $\alpha_2 = 1.36^{+0.01}_{-0.02}$. Fitting the x-ray light curve together with the optical ones, we find the same parameters from 26.6 ks onward, but to fit the early part of the X-ray light curve, we need another power-law segment with a slope of $1.29^{+0.02}_{-0.01}$ and a break at $26.6^{+4.5}_{-6.6}$ ks (Fig. 2). Therefore, from 26.6 ks onward a common description of all the optical, UV, and x-ray behavior is possible, whereas at earlier times, an extra x-ray component is required.

We interpret the early x-ray light curve (up to 26.6 ks) as the superposition of a standard afterglow (forward shock emission) and either the prolonged activity of the central engine or/and the contribution from the reverse shock emission (18–20). After 26.6 ks, the optical and x-ray light curves share the same behavior and decay slopes (Fig. 2), including a break at $t_{\text{break}} \sim 37$ ks. This achromatic break is suggestive of a jet break, although the post-break decay ($\alpha_2 = 1.36$) is shallower than predicted in the simplest theory [an increase in decay slope > 1 would be expected (21)]. This could be due to additional components contributing to the flux, to a time dependence of the microphysical parameters governing the fraction of shock energy going to electrons (ϵ_e) and magnetic field (ϵ_B), or to the fact that we observed a canonical jet not exactly on axis, but still within the jet opening angle (22, 23).

Because the optical and the x-ray emission belong to the same spectral power-law segment, it is possible to constrain the characteristic frequencies of the afterglow spectra, in turn constraining the microphysical parameters of the relativistic shock. Additional information comes from the high-energy γ -ray emission (2). The γ -ray flux above 100 MeV peaks at ~ 20 s. If this emission is due to afterglow radiation, the peak time implies a bulk Lorentz factor $\Gamma_0 \sim 500$ (2, 7). Furthermore, the presence of the giga-electron volt peak suggests a homogeneous circumburst density profile (24). Guided by these constraints in our choice of parameters, we used the BOXFIT code developed by van Eerten *et al.* (25) to model the afterglow. Rather than trying to perform a formal fit to the data, we checked whether this burst, with an unprecedented data coverage and richness, can be interpreted in the framework of the standard model for the afterglow emission, or else whether it forces us to abandon the standard framework. We gave more weight to the optical and higher energy fluxes because they carry

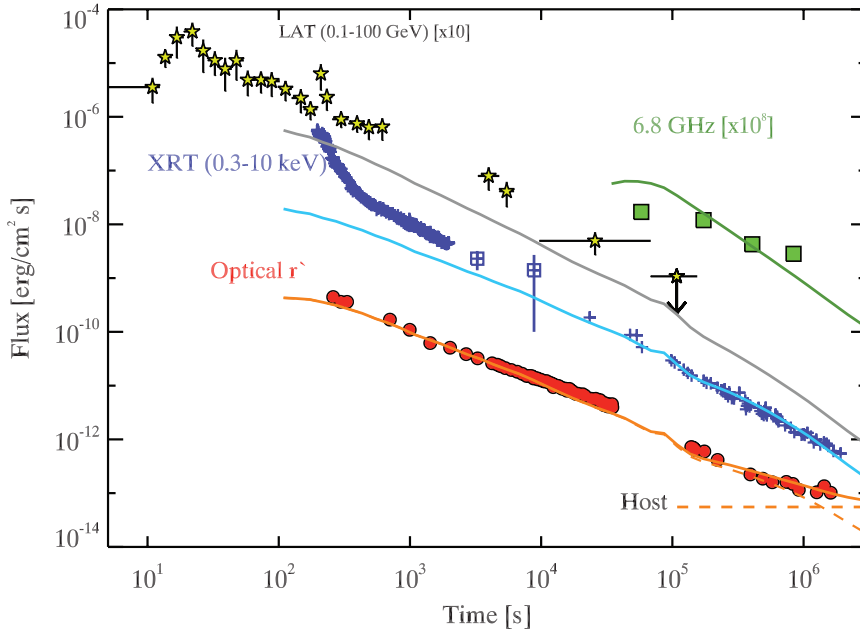


Fig. 3. Radio, optical, x-ray, and γ -ray light curves of GRB 130427A. Radio data are from (31): All measurements are taken at 6.8 GHz except the later one, at 7.3 GHz. X-ray data are the same reported in Fig. 2 (7). LAT γ -ray data are from (2). Corresponding model predictions adopt a description in terms of the van Eerten *et al.* model (25). To properly fit the radio data, only a fraction of the electrons must be accelerated after ~ 70 ks (table S10) [discussion is available in (7)].

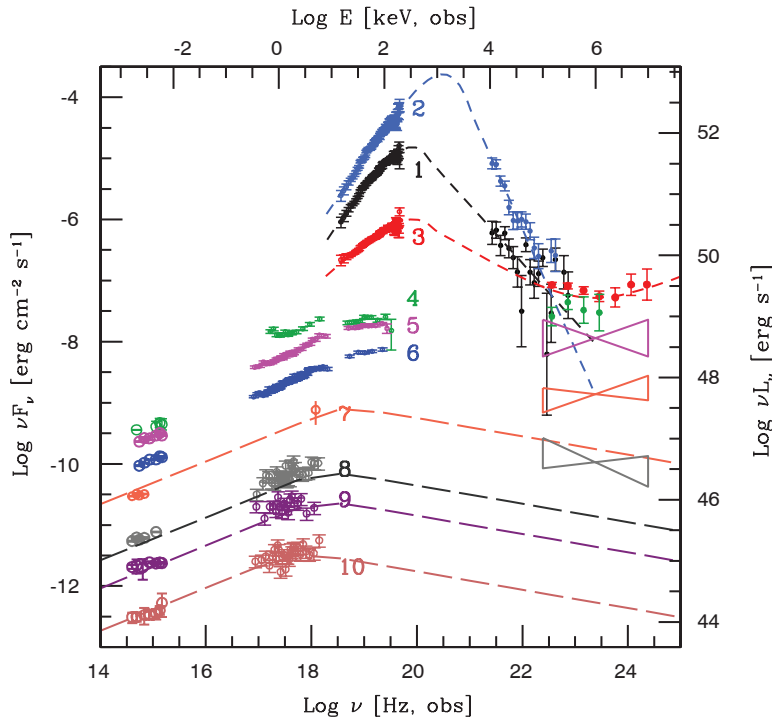


Fig. 4. Spectral Energy Distributions (SEDs) of GRB 130427A taken at different times, from the optical to the GeV bands. LAT data are from (2). For SED 1 and SED 2, the model is a Band function; for SED 3, the model is a Band + power law. Short dashed lines indicate phenomenological fits to the BAT + LAT data. Long dashed lines indicate results of the van Eerten *et al.* model (25), with parameters discussed in (7). The different SEDs refer to the following time intervals: 1, 0 to 6.5 s; 2, 7.5 to 8.5 s; 3, 8.5 to 196 s; 4, 352 to 403 s; 5, 406 to 722 s; 6, 722–1830 s; 7, ~ 3 ks; 8, ~ 23 ks; 9, ~ 59 ks; 10, ~ 220 ks (host galaxy contribution has been subtracted in this case). From the optical SED analysis, the intrinsic extinction is negligible.

most of the afterglow luminosity, which is orders of magnitude greater than the radio flux.

Neither reverse shock nor inverse Compton (IC) emission are included in the model, but this does not affect our conclusions, which primarily concern the late time synchrotron afterglow (7). The synchrotron flux predicted by the model reproduces the optical emission and the x-ray light curve after ~ 10 ks reasonably well, whereas the early x-ray flux is likely due to an additional component (Fig. 3). Our model underestimates the GeV emission, but this, given the large ϵ_s/ϵ_B ratio, can be due to synchrotron Self-Compton emission, as envisaged by (2, 26, 27). The model can also roughly reproduce the radio emission (7).

Consistent with the light curve analysis, the synchrotron flux predicted by the model reproduces reasonably well the optical and x-ray parts of the spectral energy distribution (SED) of the afterglow (Fig. 4), but it underestimates the GeV emission. Although the model does not entirely reproduce the complexity shown by the data, it does capture the main features of the emission properties in the pure afterglow phase.

Overall, the properties of GRB 130427A are similar to those of the powerful GRBs typically seen at $z \sim 1 - 2$ [a comparison is available in (7)]. This is the most powerful GRB at $z < 0.9$. It obeys the spectral energy correlations, such as the $E_{\text{peak}} - E_{\text{iso}}$ (28) correlation and the $E_{\text{peak}} - L_{\text{peak}}$ (29) correlation. Interpreting as a jet break the break observed at ~ 37 ks makes GRB 130427A consistent with the collimation corrected energy–peak energy correlation (7, 30). GRB 130427A is also associated with a supernova, extending the GRB-SN connection also to such powerful and high- z bursts. GRB 130427A stands as an exceptional example indicating that a common engine is powering these huge explosions at all powers, and from the nearby to the very far, early universe.

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Supplementary Materials

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The First Pulse of the Extremely Bright GRB 130427A: A Test Lab for Synchrotron Shocks

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Gamma-ray burst (GRB) 130427A is one of the most energetic GRBs ever observed. The initial pulse up to 2.5 seconds is possibly the brightest well-isolated pulse observed to date. A fine time resolution spectral analysis shows power-law decays of the peak energy from the onset of the pulse, consistent with models of internal synchrotron shock pulses. However, a strongly correlated power-law behavior is observed between the luminosity and the spectral peak energy that is inconsistent with curvature effects arising in the relativistic outflow. It is difficult for any of the existing models to account for all of the observed spectral and temporal behaviors simultaneously.

In the context of Gamma-ray burst (GRB) 130427A, which triggered the Gamma-Ray Burst Monitor (GBM) (1) on the Fermi Gamma-

Ray Space Telescope on 27 April 2013 at $T_0 = 07:47:06.42$ UTC (2–4) is an extreme case. The peak flux on the 64-ms time scale is $1300 \pm$

$100 \text{ photons s}^{-1} \text{ cm}^{-2}$ in the 10 to 1000 keV range and the fluence, integrated over the same energy range and a total duration of ~ 350 s, is $(2.4 \pm 0.1) \times 10^{-3} \text{ erg cm}^{-2}$. The longest continuously running GRB detector, Konus on the Wind spacecraft, has been observing the entire sky for nearly 18 years, and only one burst had a larger peak flux, by $\sim 30\%$ (GRB 110918A) (5). GRB 130427A is the most fluent burst in the era starting with the 1991 launch of the Burst and Transient Source Experiment (BATSE) on the Compton Gamma-Ray Observatory. Finally, the energy of the spectral peak in the first time bin ($T_0 - 0.1$ to 0.0 s), 5400 ± 1500 keV, is the second highest ever recorded (6).

The initial pulse (Fig. 1), lasting up to 2.5 s after the trigger, stands on its own as being so bright ($170 \pm 10 \text{ photons s}^{-1} \text{ cm}^{-2}$ peak flux for 10 to 1000 keV in the 64-ms time bin at $T_0 + 0.51$ s) as to be ranked among the 10 brightest GBM or BATSE bursts (7–9). The brightness allows us to track the spectral evolution of the rising portion of a well-separated pulse with unprecedented detail (10). Evident in the GBM low-energy light curve [Fig. 1; as well as the 15 to 350 keV light curve presented in (11)] are fluctuations starting at around 1 s that are not present at higher energies. If these represent additional low-energy pulses, their presence clearly does not dominate the analyses presented below.

Past studies of time-resolved spectra of simple pulses in GRBs indicate that there are broadly two classes of spectral evolution. These are called “hard-to-soft” and “tracking” pulses (12, 13), depending on whether the energy of the peak in the νF_ν spectrum (generically called E_{peak} herein) monotonically decays independently of the flux evolution or else generally follows the rise and fall of the flux. Typically, there are at most one or two spectra available for fitting during the rising portion of the flux history. What makes this event unique is that there are roughly six time bins with excellent counts statistics before the peak in the 10 to 1000 keV flux.

As seen in Fig. 1, there is a clear trend in the individual detector's light curves: the >20 MeV

Fermi Large Area Telescope (LAT) low-energy (LLE) (14, 15) light curve peaks before the GBM trigger time (T_0), whereas the GBM bismuth germanate (BGO) detector #1 (300 keV to 45 MeV) and sodium iodide (NaI) detector #6 (8 to 300 keV) peak at successively later times. To quantify this, we performed an energy-dependent pulse lag analysis using a Discrete Cross Correlation Function (DCCF) and obtained the time lags τ (16) between the highest energy LAT LLE light curve and light curves at several selected energy ranges in the GBM NaI and BGO detectors (Fig. 1, inset). We find good agreement between the expected lag behavior and the pulse width model $W(E) \propto E^\alpha$ (17), obtaining a fitted value for $\alpha = -0.27 \pm 0.03$ (18). This model was previously fit to 400 pulses from 41 BATSE GRBs (17); an average value of $\alpha = -0.41$ was found. Synchrotron shock model simulations made by Daigne and Mochkovitch (19) found $\alpha \sim -0.4$ for pulses of 2- to 10-s duration, but $\alpha > \sim -0.2$ for pulses of 0.1 to 1 s. Three LAT photons with energies greater than 100 MeV are clustered in coincidence with the LLE peak, and so may arise by the same mechanism.

Although most GRB spectra are well fit by the smoothly joined broken power-law function of Band *et al.* (20), in some cases the simultaneous fit of a Band function together with an additional blackbody component is significantly better statistically (6, 21, 22). Burgess *et al.* (23, 24) also noted the requirement of an additional black-

body component but replaced the phenomenological Band function with a physically motivated synchrotron function. We present two separate time-resolved spectral analyses for the first 2.5 s of GRB 130427A, with comparable goodness of fit: the Burgess *et al.* synchrotron function plus blackbody, and the Band function (18). A blackbody component is not required when the more flexible Band function alone is used. Although the time evolution of E_{peak} as determined by Band function fits is consistent with a single power law (with an index of -0.96 ± 0.02), the evolution of the synchrotron peak energy is not (Fig. 2). A broken power-law fit is better constrained and shows a shallower decay before the pulse peak, with an index of -0.4 ± 0.2 during the rising phase and -1.17 ± 0.05 during the decaying phase and a fitted break time at $T_0 + 0.28 \pm 0.08$ s, or ~ 0.2 s before the pulse peak in the 10- to 1000-keV flux. Both fitted indices during the decay phase are consistent with the -1 power-law index expected from standard fireball curvature effects (25, 26). The shallower spectral peak decay index before the light curve decay phase has a natural explanation in the context of the pulse being driven by a shock between thick, colliding shells (19).

Two-component models including a thermal contribution (27) constrain the value of the photospheric radius using the blackbody flux and temperature (kT) (see table S1). Comparing with the flux of the dominant nonthermal spectral component then permits determination of the Lorentz

factor at the photosphere (Γ_{ph}) (28). As shown in Fig. 3, the minimum value of the photospheric bulk Lorentz factor Γ_{ph} starts out at 500 and monotonically decreases to ~ 100 over the duration of the pulse (similar to behavior observed in GRB 110721A) (29). Internal shocks require higher Lorentz factors at later times. However, this might still be consistent with the monotonically decreasing Γ_{ph} if the outflowing shell that produces this photospheric component produced the nonthermal triggering pulse by colliding with a slower and slightly earlier ejected shell that did not produce detectable photospheric emission. Otherwise, the observed behavior would favor magnetic reconnection models or mini-jets (30, 31), which abandon a simple spherical geometry.

Using the measured redshift of $z = 0.34$ (32), the host rest-frame luminosity and synchrotron peak energies are calculated, and the decay-phase apparent isotropic luminosity $L - E_{\text{peak}}$ correlation is fit with a power-law index of 1.43 ± 0.04 (Fig. 4). A theoretical analysis of high-latitude curvature radiation produced in relativistic shell collisions of spherical blast waves shows a $L \propto E_{\text{peak}}^3$ during the decay phase of a pulse (25, 26), contrary to the behavior shown in Fig. 4. In a picture of an expanding fluid element rather than a colliding shell, synchrotron emission by electrons with characteristic energy γ_e obeys the relations $E_{\text{peak}} \propto \Gamma B \gamma_e^2$ and $L \propto \Gamma^2 B^2 \gamma_e^2$, for γ_e and B both in the jet frame. In the optically thin coasting phase of the outflow, the bulk Lorentz factor Γ

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is constant. Naively assuming that the magnetic flux is frozen in the flow ($BR^2 \propto \text{const.}$ in the comoving frame, where R is the comoving emis-

sion region radius), then adiabatic losses of the electrons imply $\gamma_e \propto R^{-1}$. A short calculation then gives $L \propto E_{\text{peak}}^{3/2}$, which is consistent with

the 1.43 index derived from the data. A constant expansion velocity dR/dt scenario predicts, however, $E_{\text{peak}} \propto R^{-4} \propto t^{-4}$. Other jet-wind assumptions

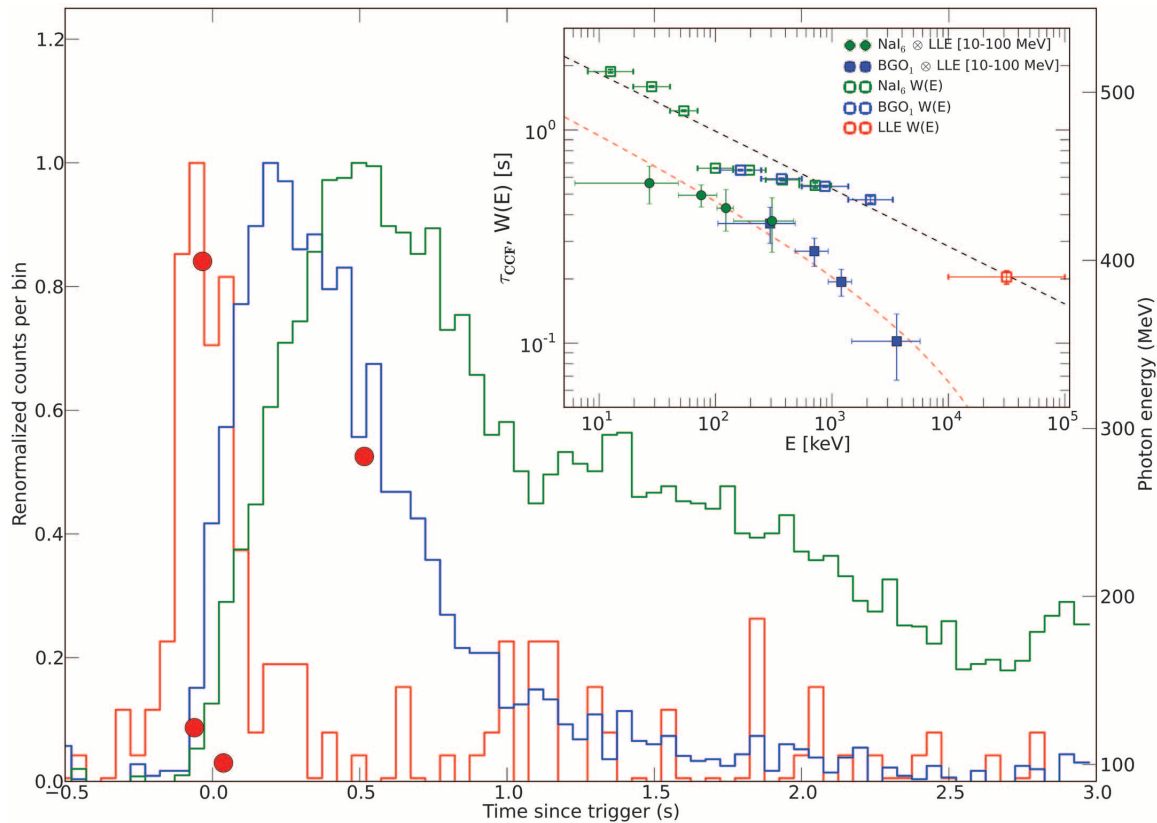
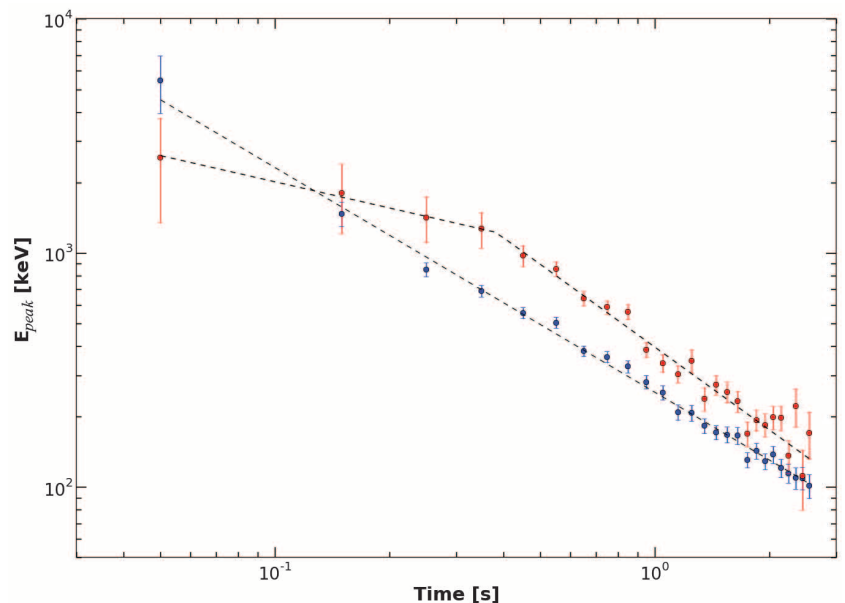


Fig. 1. The first 3 s of GRB 130427A. Shown, are composite light curves for the three Fermi detector types. Green, GBM NaI #6 (10 to 300 keV); blue, GBM BGO #1 (300 keV to 45 MeV); red, LAT LLE (>20 MeV). Each curve has been normalized so that their peak intensities match. High-probability LAT photons >100 MeV are indicated by circles (right axis, energy in MeV). (Inset) Lag analysis of the triggering pulse of GRB 130427A. Time lag τ (filled symbols) as determined by the DCCF

analysis between the (10 to 100 MeV) LLE light curve and selected energy bands of the NaI (green) and BGO (blue) light curves. Also displayed are fitted pulse widths as a function of energy $W(E)$ (hollow symbols, in seconds) for several energy bands. The two dashed lines represent: 1, the best-fit power-law model (χ^2 of 5.6 for 9 degrees of freedom) for $W(E)$ (black), and 2, the expected dependence of the time lag τ as a function of energy (red), assuming the same power-law index as in 1.

Fig. 2. The fitted Band function E_{peak} (blue) and synchrotron peak energies (red) as a function of time. The times are referenced from when the LLE light curve peaks 0.1 s before the trigger. A broken power-law fit to the red points is indicated by a dashed line (early time decay index of -0.4 ± 0.2 , with a break at 0.38 ± 0.08 s, breaking to an index of -1.17 ± 0.05 with a $\chi^2 = 28$ for 22 degrees of freedom). We also show the Band function E_{peak} values for the same time intervals with a single fitted power-law index (-0.96 ± 0.02 with a χ^2 of 19 for 24 degrees of freedom).



yield different correlations—for example, for the deceleration epoch where $d\Gamma/dt < 0$ or for radial field evolution appropriate for jet cores.

The isolated initial pulse of GRB 130427A is apparently unmodified by preceding engine activity or nascent external shock emission. Our analysis shows that there is good agreement between

the pulse width as a function of energy and the expected lag, that the characteristic energy has roughly a -1 power-law decay with time during the decaying phase, that the temperature of the blackbody component implies a photospheric radius incompatible with the internal shock radius, and that the apparent isotropic luminosity is re-

lated to the $3/2$ power of the characteristic energy. It is a challenge to explain all these behaviors simultaneously.

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Supplementary Materials

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Supplementary Text

Table S1

References (33–35)

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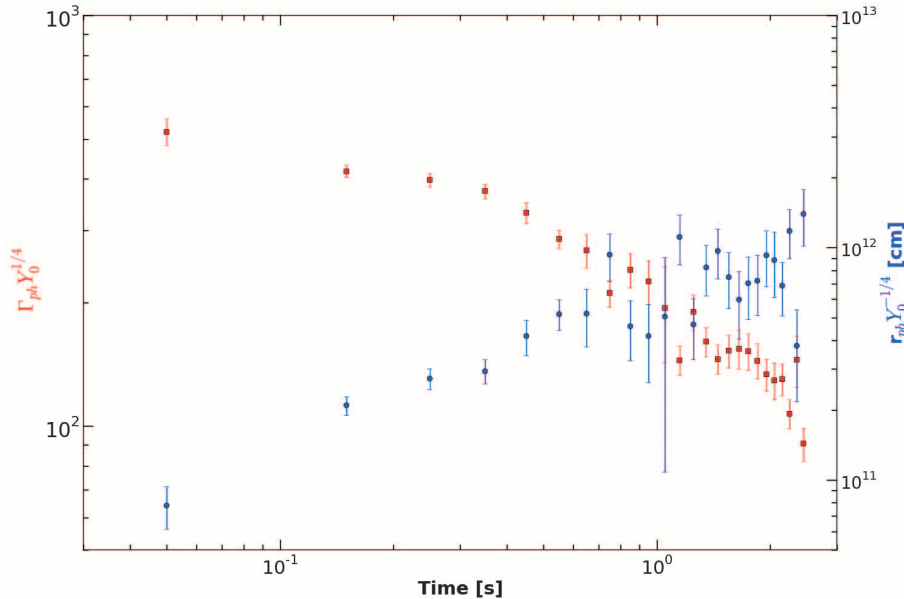


Fig. 3. Plot showing trends in the derived photospheric Lorentz factor (red, left axis) and radius (blue, right axis). The reference time is the same as in Fig. 2. We obtain both values from the instantaneous ratio of the observed blackbody component flux to the total flux, following equations 4 and 5 in (28) and assuming a value of 1 for the ratio between the total emitted thermal energy versus the total energy emitted in gamma rays (γ_0).

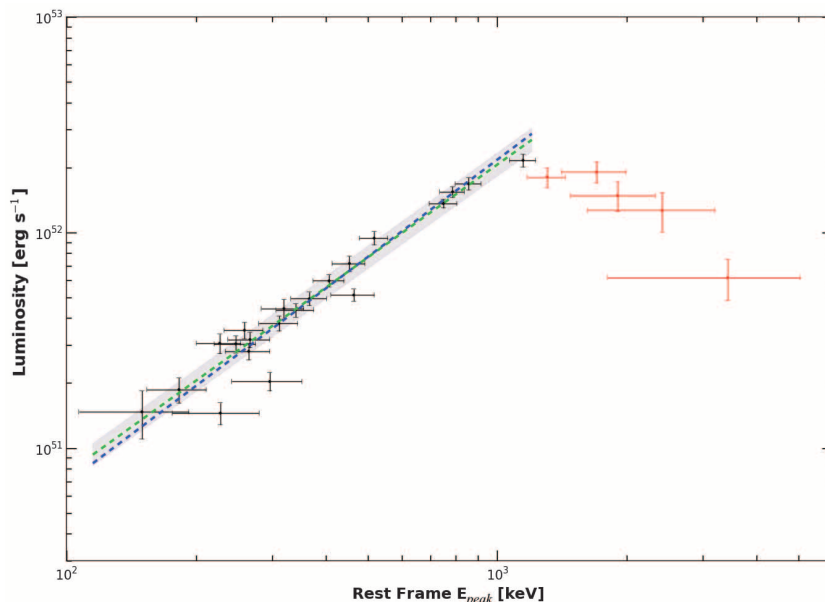


Fig. 4. Correlation between the GRB 130427A host rest frame synchrotron peak energy and isotropic luminosity during the rising phase (red) and decaying phase (black) of the triggering pulse. Time progresses approximately from right to left on the plot. The 1.43 ± 0.04 power-law index fit to the black points is shown in green (region of uncertainty in gray), and the $3/2$ power law from the magnetic flux-freezing calculation in the text is indicated in blue.

Exciton Liquid in Coupled Quantum Wells

Michael Stern,*† Vladimir Umansky, Israel Bar-Joseph

Excitons in semiconductors may form correlated phases at low temperatures. We report the observation of an exciton liquid in gallium arsenide/aluminum gallium arsenide-coupled quantum wells. Above a critical density and below a critical temperature, the photogenerated electrons and holes separate into two phases: an electron-hole plasma and an exciton liquid, with a clear sharp boundary between them. The two phases are characterized by distinct photoluminescence spectra and by different electrical conductance. The liquid phase is formed by the repulsive interaction between the dipolar excitons and exhibits a short-range order, which is manifested in the photoluminescence line shape.

More than 40 years ago, it was shown theoretically that excitons in semiconductors may form a condensate at cryogenic temperatures (1). Subsequently, a wealth of classical and quantum phases, ranging from electron-hole plasma to superfluid liquid, have been predicted to occur in this material system (2–4). Spatially indirect dipolar excitons with the electrons and holes residing in separate coupled quantum wells (CQWs) offer a promising test bed for these phenomena (5–8). They are characterized by a strong repulsive dipole-dipole interaction, which can be easily engineered by designing the sample structure. Their density, n , and the distance between the centers of the wells, d , are key parameters that govern the formation of condensed states in this system. Together they set the strength of the dipole interaction with the surrounding carriers. The interaction energy, $\delta E(n) = \iint V(r)n(r)d^2r$, can be expressed in terms of an effective radius, r_0 , around every exciton, within which the carrier density is depleted as a consequence of the repulsive interaction V

$$\delta E(n) = \frac{\langle n \rangle e^2}{\epsilon} \left[\sqrt{r_0^2 + d^2} - r_0 \right] \quad (1)$$

Here, $\langle n \rangle$ is the average density, e is the electron charge, and ϵ is the dielectric constant. $\delta E(n)$ can be directly measured in a photoluminescence experiment as an energy shift of the recombination energy with density.

Theory predicts that at densities well above the Mott transition, the system may form an exciton liquid (3, 4). For d smaller than the exciton Bohr radius a_B , the exchange interaction can compensate for the dipole repulsion, and a quantum liquid is formed. For $d \geq a_B$ this phase is not stable, but the system may still form a classical liquid. The origin of this classical liquid is related to the formation of a region around each exciton,

with radius $\rho \approx 4d^2/a_B$, where the wavefunctions of other excitons are exponentially small (4). When $n\rho^2 \geq 1$, the wave functions of neighboring excitons do not overlap, and quantum correlations are suppressed. Classical correlations, on the other hand, are very strong and may give rise to short-range order. This classical limit of strongly interacting dipoles is the focus of our work.

The system we studied was formed by two gallium arsenide (GaAs) quantum wells with well widths of 12 and 18 nm, separated by a 3-nm $\text{Al}_{0.28}\text{GaAs}$ barrier (energy diagram shown in Fig. 1A); the distance between the centers of the wells is thus $d = 18$ nm, almost twice as large as a_B . The carriers were photogenerated by a laser and separated by the application of a gate voltage V_g in the direction perpendicular to the wells. More experimental details are given in (9).

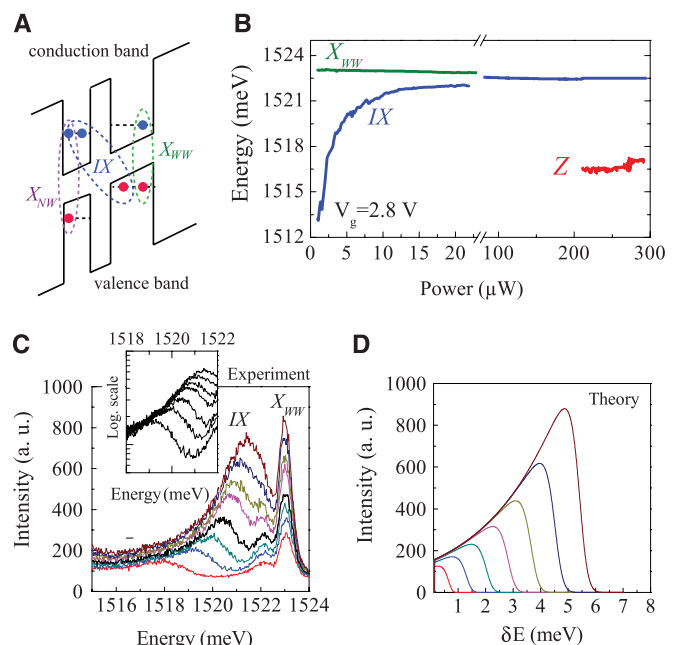
In Fig. 1B, we show the evolution of the spectrum with increasing laser power P . Between

1 and 100 μW , the indirect recombination energy (IX) increases monotonically with P as the density n increases. This shift to higher energy is the manifestation of the growing interaction energy, $\delta E(n)$, and it allows us to determine the electron-hole density: For example, at 20 μW , $\delta E = 9\text{meV}$, which corresponds to $\sim 2 \times 10^{10}$ to $3 \times 10^{10} \text{ cm}^{-2}$. δE is not linear with power and saturates as the IX peak approaches the wide-well exciton line, X_{WW} . This behavior can be understood when it is noted that the recombination rate Γ is proportional to the overlap integral of the electron and hole wavefunctions (10): As the density increases, the potential drop between the quantum wells is screened, and Γ increases exponentially. An interesting implication of this behavior is that at $P \gg 20 \mu\text{W}$, the density across the sample is almost constant and depends only weakly on the local power.

In Fig. 1C, we show the spectra at various powers, where the blueshift of the IX peak with power is clearly evident. The IX spectra at all powers are characterized by a pronounced exponential tail at the low-energy side (11). This line shape reflects the broad range of interaction energies (and corresponding r_0 values) that can be realized in an electron-hole plasma in CQWs. We find that the exponential tails of the lines at low and medium powers merge with the high-power spectra. Indeed, one can reproduce the essential features of the observed behavior by calculating the interaction of a recombining dipole with the surrounding gas, taking into account the change of Γ with energy (Fig. 1D). Hence, this low-energy tail can be viewed as the spectral signature of interacting electron-hole plasma in CQWs. More details are given in section 1.1 of (9).

Fig. 1. The evolution of the photoluminescence spectrum with power. (A)

The energy band diagram of the asymmetric CQW under a perpendicular applied electric field. X_{WW} is the exciton in the wide well, X_{NW} is the exciton in the narrow well, and IX is the interwell indirect recombination. The blue circles represent electrons and the red circles represent holes. **(B)** Photoluminescence peak positions for X_{WW} , IX , and Z lines as a function of laser power at $T = 1.5$ K. **(C)** The evolution of the spectrum with power between 3 and 10 μW (the small peak at 1522 meV is the trion). The inset shows the spectra on a logarithmic scale. a.u., arbitrary units. **(D)** Calculated spectra demonstrating the formation of the low-energy exponential tail.



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At higher power, above $P = 210 \mu\text{W}$, a new line, denoted by Z, appears below the IX line and shifts only slightly with power. One can show that this line is also due to the indirect recombination of electrons in the narrow well with holes in the wide

well. It shifts linearly with the gate voltage, approximately parallel to the IX line, remaining 3 to 4 meV above E_{IX}^0 , the IX energy at low power, $<1 \mu\text{W}$.

We found that the Z line came from a well-defined spatial region in the illuminated area. We

imaged the photoluminescence from the mesa and observed that below threshold, the image is smooth and follows the Gaussian profile of the excitation spot. Above threshold, a striking behavior appears: The luminescence separates into two regions, with a narrow (resolution-limited) dark border between them (Fig. 2A). The location of this boundary is unrelated to any structural parameter of the sample and can be affected by slight shifts of the exciting laser spot (movies S1 and S2).

The spectral properties of the two regions were analyzed by local photoluminescence measurements, with a $5\text{-}\mu\text{m}$ spatial resolution (Fig. 2B). As we moved across the boundary, the spectrum changed from being dominated by IX in the region denoted as I in Fig. 2A to being dominated by Z in region II.

In the following, we show that the Z line is a manifestation of an exciton liquid.

1) Criticality. The transition of the system into the phase-separated state is thermodynamic and occurs below a critical temperature and above a threshold power. Figure 2C shows the relative area of region II as a function of temperature (T). This phase appears abruptly at temperature $T = 4.7 \text{ K}$. Figure 2D demonstrates the power dependence: The appearance of region II above a threshold power is clearly seen. In this case, the area of region II extends gradually until it covers the entire sample.

2) High density. Time-resolved measurements reveal that the lifetime of the carriers in region II is ~ 60 to 80 ns (Fig. 2E), which is approximately twice as large as in region I. The phase separation therefore manifests two density regions: region I with lower density and region II with higher density. Using the absorption value of the CQW (12), we can estimate the density in region II to be $\sim 4 \times 10^{10}$ to $6 \times 10^{10} \text{ cm}^{-2}$ (9). At this high density, we get $np^2 \approx 10$, fulfilling the

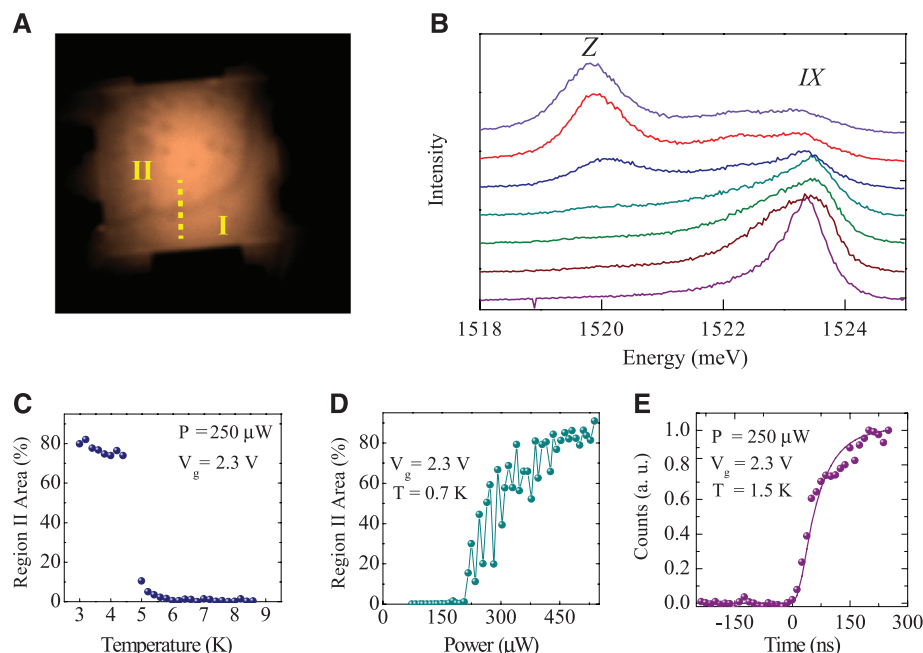


Fig. 2. The phase separation. (A) Image of the photoluminescence from the mesa showing the phase separation between region I and region II. The dark spots correspond to defects in the sample, which give reduced luminescence intensity in region II. This measurement was performed at $T = 1.5 \text{ K}$, $V_g = 2.3 \text{ V}$, and $P = 250 \mu\text{W}$. (B) Spectra measured along the dotted yellow line in (A) in the vicinity of the phase boundary. Each spectrum is shifted vertically by a fixed value, corresponding to its location along the line. (C and D) Evolution of the area of region II as a function of temperature (C) and laser power (D). The noise in the measurements reflects the fact that at some power levels, especially near threshold, the phase boundary exhibits large temporal fluctuations. (E) The rise of the Z line as a function of time (t) measured by correlated photon counting using an acousto-optic modulator. At $t = 0$, the laser is switched on and persists for $\sim 4 \mu\text{s}$. The solid line shows a fit to the experimental data, which gives a lifetime of $70 \pm 10 \text{ ns}$.

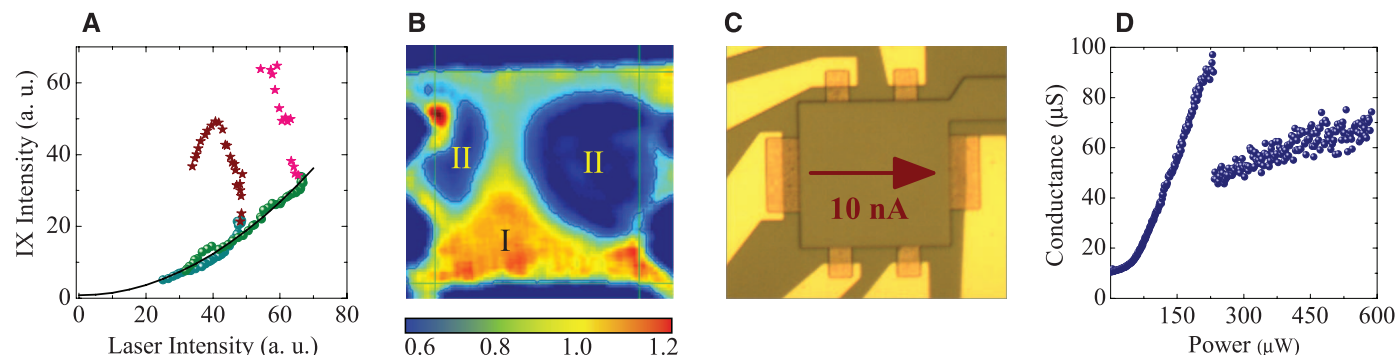


Fig. 3. The low diffusivity of region II. (A) A measurement of the local IX intensity as a function of the local laser intensity along two lines, similar to the dotted line in Fig. 2A. The brown and pink stars were measured in region I; the blue and green dots were measured in region II. The parabolic solid line demonstrates the correlation between the IX and laser intensities in region II. (B) The ratio between the photoluminescence images taken before and after phase separation. The thin green lines indicate the position of the mesa and are guides for the eyes. The blue color shows a reduction of intensity (<0.75)

after phase separation, whereas the red color represents an increase (>1.1). The diffusion away from the phase separation line toward the edges of the sample is clearly seen in region I. (C) Optical microscope image of the sample showing the four-probe electrical contacts to the $100\text{-}\mu\text{m}$ -square mesa. (D) Four-probe conductance as a function of laser power at $T = 1.5 \text{ K}$, $V_g = 2.3 \text{ V}$. The abruptness of the drop is due to the fact that the time interval between the measured points was 1 s , which is shorter than the nucleation time at $200 \mu\text{W}$ (9).

condition for a classical liquid, $n\rho^2 \gg 1$. The Z line energy is shifted relative to E_{IX}^0 by $\delta E = 3$ to 4 meV only. Using Eq. 1, we find that this corresponds to $r_0 \approx 25$ to 35 nm, which is approximately half the average interparticle distance at these densities.

3) Low diffusivity. Figure 3A shows the local intensity of the IX line as a function of the local excitation power. The IX intensity is well correlated with the excitation power in region II and is uncorrelated with it in region I. This implies that in region I, the carriers can recombine away from where they were excited, whereas in region II, they are localized. The high diffusivity of the carriers in region I is also seen when we compare the luminescence intensity profile in this region before and after the phase separation (Fig. 3B). The carriers in region I are pushed away from the region boundary and aggregate at the sides of the mesa.

The localization of carriers in region II is corroborated by in-plane electrical transport measurements conducted in a four-probe geometry (Fig. 3C). Figure 3D shows the conductance of the sample, σ , as a function of power. The onset of phase separation is clearly manifested by a drop of the conductance value and a reduction of the slope of the curve. Above the threshold power, the sample is divided into two regions, each with a different conductance, with $\sigma_I \gg \sigma_{II}$ (see materials and methods in the supplementary materials). The existence of a high-density region with low conductance is consistent with an exciton liquid.

4) Ordering. An important insight comes from studying the line shape of the Z line (Fig. 4A). The characteristic low-energy tail of the electron-hole plasma is absent, and the line is an almost perfect Gaussian (Fig. 4B). Noting that the low-energy tail is a manifestation of multiple values of r_0 , which characterize the gas phase, we are led to conclude that the Gaussian shape represents a narrow distribution around a dominant configuration. We used a local photoluminescence

measurement to extract the Z line at various locations and study its linewidth dependence on density. We found that the width of this Gaussian line decreases with increasing density (Fig. 4C). This line's narrowing cannot be explained by an uncorrelated gas, which should cause the linewidth to grow with density (13). To explain this behavior, we conducted a simple Monte Carlo calculation, assuming a fixed depletion region of radius r_0 around each exciton. With this assumption, the calculation reduces to randomly inserting N dipoles on a grid with r_0 periodicity and calculating their interaction energy. We found that the linewidth is indeed Gaussian and decreases with increasing density when $n\rho r_0^2 \geq 1/2$. Above this density, the number of possible configurations starts to decrease, giving rise to a narrower range of interaction energies. In fact, by taking $r_0 \approx 25$ nm, the value obtained from the estimated density, we get a quantitative agreement with the observed behavior (Fig. 4C).

The accumulation of these findings indicates that a strongly correlated ordered phase appears below a critical temperature and above a threshold density. The sample separates into two phases: an interacting electron-hole plasma in region I and a liquid phase in region II. Region II should not be perceived as a continuous liquid phase. The fact that we observe both Z and IX lines in region II (Fig. 2B) indicates that this region consists of a mixture of droplets and gas. Indeed, the existence of such a mixture was predicted by calculating the free energy of an exciton gas and solid (14).

Several other experimental findings are consistent with this interpretation. The first is the existence of a dark boundary between the two phases (Fig. 2A). This unusual behavior is explained by the fact that the carriers in the gas region experience a strong repulsive force from the localized high-density dipoles at the boundary. This repulsion creates a depletion region at their interface and induces a current of electron-

hole pairs toward the sides of the mesa (Fig. 3B). Another item of supporting evidence is related to the phase separation dynamics. We found clear evidence for a nucleation process: There is a relatively long (seconds) delay time between the onset of the laser light and the appearance of the phase separation (fig. S3). After this delay time, region II spreads from a corner of the mesa to its full extent (movie S3).

In closing, we wish to point out an unexplained observation. By comparing the integrated photoluminescence intensity before and after the phase separation, we find that the recombination in region II is not entirely radiative, and approximately 15% of the intensity is lost. Furthermore, we find that this darkening disappears at a weak perpendicular magnetic field, $B \sim 0.3$ T. Above this field, the phase separation is still observed. The fact that the phase separation can be observed without darkening rules out the formation of a dark condensate as the source of the observed behavior (8, 15). Further studies are needed to examine this behavior.

References and Notes

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Supplementary Materials

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Materials and Methods

Supplementary Text

Figs. S1 to S5

References (16–18)

Movies S1 to S3

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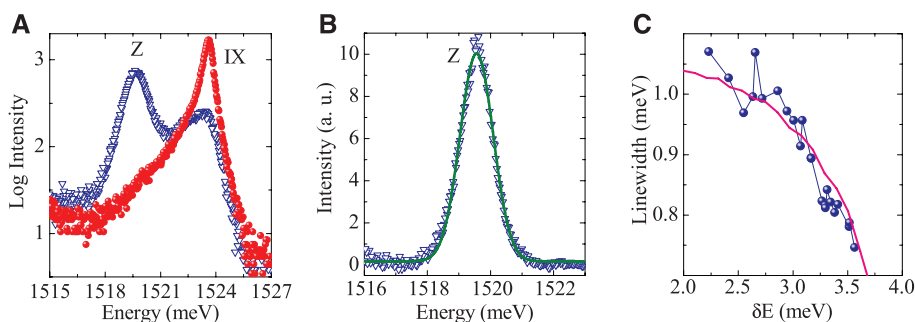


Fig. 4. Shape and width of the Z line. (A) A comparison between the photoluminescence spectrum in region I (red) and region II (blue). (B) The spectrum of the Z line obtained after removal of the exponential energy dependence, $\Gamma(E)$. The solid green line shows an excellent fit to a Gaussian. (C) The width of the Z line as a function of the energy shift δE . The reduction of the line width with density is clearly seen. The solid red line shows the width obtained by Monte Carlo simulations, taking into account 0.4 meV of inhomogeneous broadening (half width at half maximum). A good agreement between measurement and calculation is obtained for $r_0 = 25$ nm.

First-Photon Imaging

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Imagers that use their own illumination can capture three-dimensional (3D) structure and reflectivity information. With photon-counting detectors, images can be acquired at extremely low photon fluxes. To suppress the Poisson noise inherent in low-flux operation, such imagers typically require hundreds of detected photons per pixel for accurate range and reflectivity determination. We introduce a low-flux imaging technique, called first-photon imaging, which is a computational imager that exploits spatial correlations found in real-world scenes and the physics of low-flux measurements. Our technique recovers 3D structure and reflectivity from the first detected photon at each pixel. We demonstrate simultaneous acquisition of sub-pulse duration range and 4-bit reflectivity information in the presence of high background noise. First-photon imaging may be of considerable value to both microscopy and remote sensing.

The capture of three-dimensional (3D) structure and reflectivity using an active imager when the back-reflected optical flux reaching the detector approaches a few photons per pixel has many applications (1–3). Imagers for such applications typically use Geiger-mode avalanche photodiodes (APDs), which can time-resolve single-photon detections with jitters of a few tens of picoseconds (4). Transverse spatial resolution is typically obtained point by point by using raster-scanned illumination and a single detector (5) or with floodlight illumination and a spatially resolving detector array (6).

In 3D light detection and ranging (LIDAR) systems (3, 7), the scene is illuminated with a stream of laser pulses, the back-reflected light is detected with a Geiger-mode APD, pixel-by-pixel range information is obtained from histograms of the time delays between transmitted and detected pulses (8), and pixel-by-pixel relative reflectivity is found from the number of photons detected in a fixed dwell time. Tens of photon detections per pixel suffice for accurate range imaging when background light is inconsequential, despite the Poisson noise inherent in photon counting (9, 10). Hundreds of photons, however, are needed for accurate reflectivity imaging, even in the absence of background light. Similar numbers are necessary for range imaging when detections arising from background light lead to anomalous range values (11, 12). In first-photon imaging, we capture 3D spatial structure and reflectivity by using only the first photon detection at each pixel. It is a computational method for low-flux imaging that produces high-quality range images, despite the presence of high background noise, and high-quality reflectivity images, when a conventional reflectivity image built from one photon detection per pixel would be featureless. These results

derive from exploiting the spatial correlations present in real-world scenes within a computational framework matched to the physics of low-flux measurements.

For each pixel, our imager used the number of illumination pulses before the first photon detection as an initial reflectivity estimate. Poisson noise precludes these pixel-by-pixel estimates from providing a high-quality reflectivity image. We suppressed that noise by exploiting the high degree of spatial correlation in real-world scenes; that is, that neighboring pixels have strong distance and reflectivity correlations punctuated by sharp boundaries. Such correlations can be captured through a Markov random field (13) or sparsity in the scene's discrete wavelet transform (DWT) coefficients (14, 15). We suppressed Poisson noise in the

reflectivity image by means of a DWT-based regularization. We also exploited spatial correlations to censor anomalous range values from an initial pixel-by-pixel range image.

Our setup (Fig. 1) consists of a pulsed laser source illuminating a scene with quasi-Lambertian reflectivity in a raster-scanned manner and an incandescent lamp that injects background light. Back-reflected laser light plus background light was collected by a Geiger-mode APD providing time-resolved single-photon detections. Each spatial location (pixel), (x, y) , was illuminated with a periodic stream of laser pulses until the first photon was detected. We recorded the first detected photon's arrival time, $t(x, y)$, relative to the most recently transmitted pulse, along with the number of pulses, $n(x, y)$, that were transmitted before that detection. The lamp's optical power was adjusted so that each first-photon detection had about 50% probability of originating from background light.

The first-photon data were used to reconstruct scene reflectivity, $\alpha(x, y)$, and 3D spatial structure, $Z(x, y)$, via the following three-step procedure. First, we connected the statistics of $n(x, y)$ to $\alpha(x, y)$. Let S be the average photon number in the back-reflected signal received from a single laser pulse illuminating a unity-reflectivity spatial location, B denote the arrival rate of background photons at the detector (16), T_r be the pulse repetition period, and γ be the imager's photodetection efficiency. The probability of not detecting a photon when pixel (x, y) is illuminated by a single laser pulse is (16)

$$P_0(x, y) = e^{-\gamma[\alpha(x, y)S + BT_r]}$$

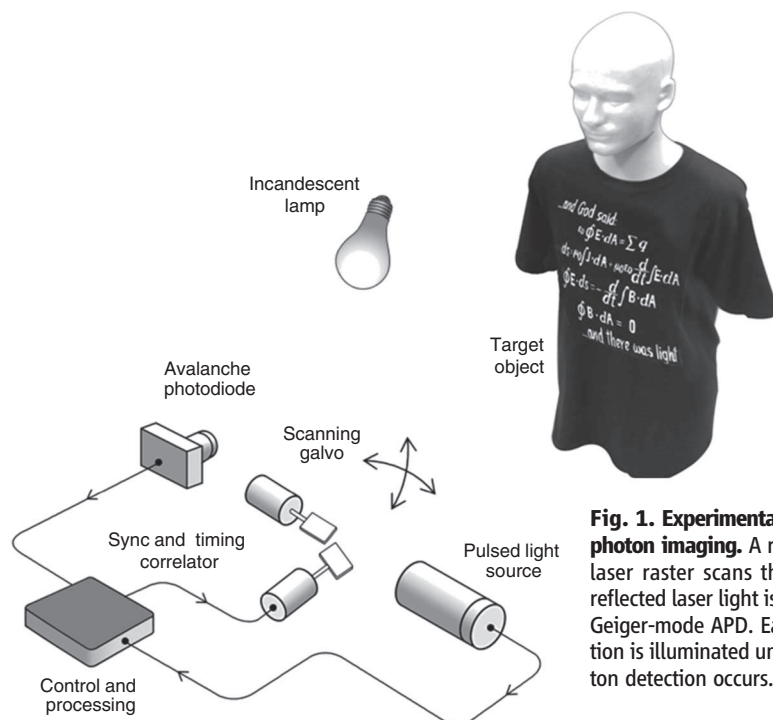


Fig. 1. Experimental setup for first-photon imaging. A repetitively pulsed laser raster scans the scene. Back-reflected laser light is detected with a Geiger-mode APD. Each spatial location is illuminated until the first photon detection occurs.

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Because each transmitted pulse gives rise to independent Poisson noise, $n(x, y)$ has the geometric distribution

$$P[n(x, y) = k] = P_0(x, y)^{k-1} [1 - P_0(x, y)],$$

$$\text{for } k = 1, 2, \dots \quad (1)$$

In the absence of background light, the pointwise maximum-likelihood reflectivity estimate, $\hat{\alpha}_{\text{ML}}(x, y)$, is proportional to $1/n(x, y)$ for $n(x, y) \gg 1$ (16). However, background illumination severely corrupts these pointwise estimates (Fig. 2, A to C). Our imager exploits spatial correlations to accurately reconstruct scene reflectivity by maximizing the product of data likelihoods (Eq. 1) over all spatial locations combined with a sparsity-promoting regularization function, namely the sum of absolute values of the image's DWT coefficients (16). The resulting global optimization problem is strictly convex (16); thus, a unique optimal reflectivity estimate is readily obtained from standard numerical solvers (17, 18).

The pointwise range estimate from a first-photon detection is $\hat{Z}(x, y) = ct(x, y)/2$ for a

transmitted pulse whose peak is at time $t = 0$, where c is the speed of light. Its root mean square (RMS) estimation error is $(c/2)\sqrt{(T_p^2 + T_r^2)/12}/2$ in the presence of background light (16), where $T_p \ll T_r$ is the laser pulse's RMS time duration and the $1/12$ factor comes from the uniformly distributed nature of the background-generated detections. Direct application of a spatial-correlation regularization to maximizing time-of-arrival likelihoods is infeasible, because background light makes the optimization objective function multimodal. Background light also causes pointwise methods to fail (Fig. 2, A to C), so we used spatial correlations to censor $t(x, y)$ values that are range anomalies.

Anomalous detections have arrival times that are uniformly distributed over the time interval $[0, T_r]$ and mutually independent over spatial locations, so that they have high variance $T_r^2/12$ relative to that of back-reflected laser-pulse (signal) detections, which are temporally concentrated and spatially correlated. Let $s(t)$ be the normalized $[\int_0^{T_r} s(t) dt = 1]$ photon-flux waveform of the laser pulse emitted at $t = 0$. At low optical flux, that is, when $\gamma(S + BT_r) \ll 1$, and the first photon detected from location (x, y) is a

signal photon, then the probability density function for $t(x, y)$ is (16)

$$p_{t(x, y)}(\tau) = s(\tau - 2Z(x, y)/c), \text{ for } 0 \leq \tau \leq T_r \quad (2)$$

which has variance $T_p^2 \ll T_r^2/12$, regardless of the reflectivity at (x, y) . The high degree of spatial correlation in the scene's 3D structure then implies that signal-generated photon arrival times have much smaller conditional variance, given data from neighboring locations, than do anomalous detections. Step 2 of the computational imager uses this statistical separation to censor background-generated arrival times from an initial pixel-by-pixel range image.

For each spatial location, a rank-ordered absolute differences statistic (19) is computed by using the photon arrival times of its eight nearest neighbors (16). Then, a binary hypothesis test—whose decision threshold is dependent on the reflectivity estimate from step 1—identifies, with high probability, whether the photon detection was due to signal or background (Fig. 2, G to I), allowing us to delete nearly all anomalous range values.

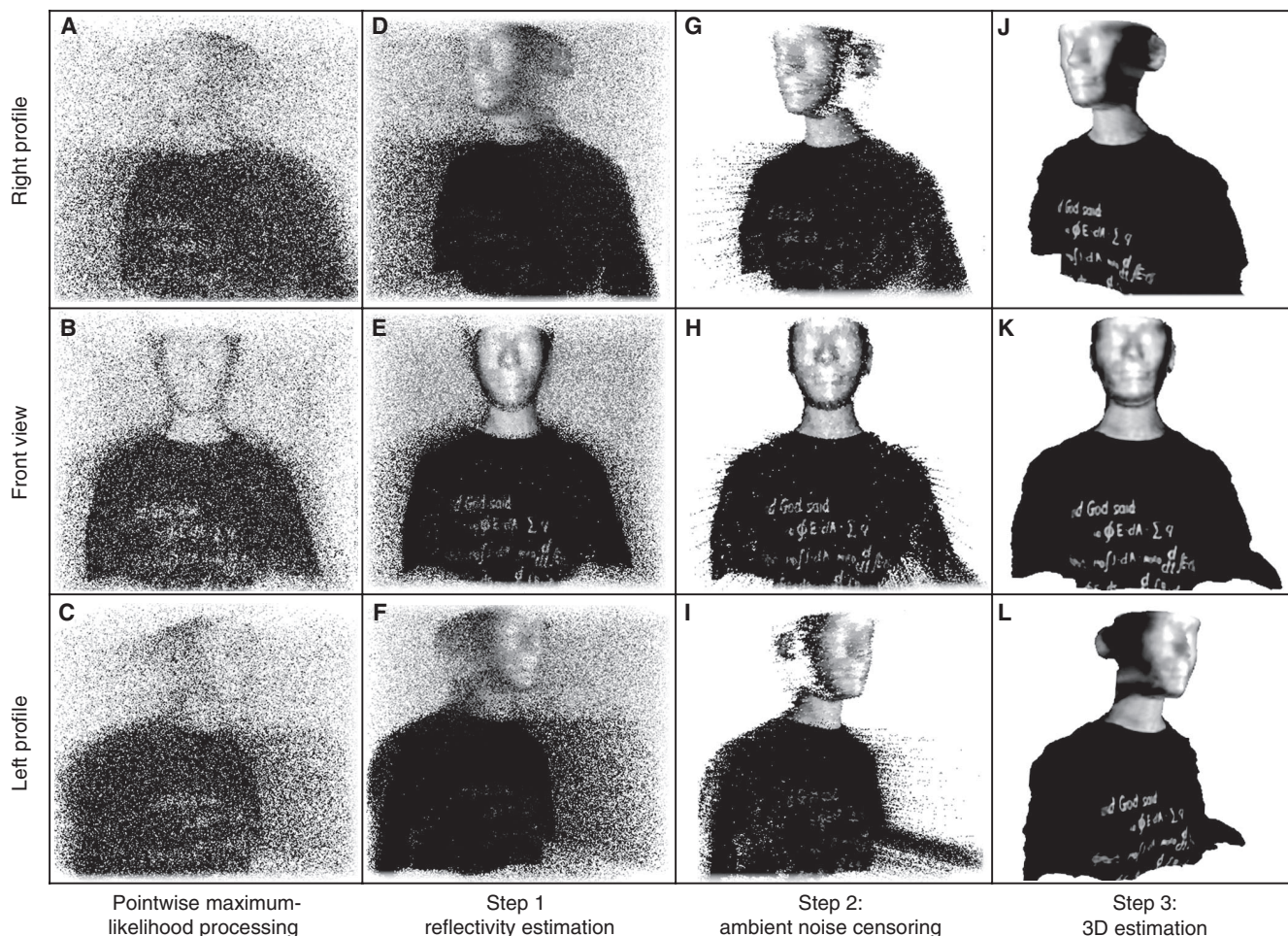


Fig. 2. Computational first-photon 3D and reflectivity reconstruction. (A to L) 3D estimates of front views and lateral views rendered as point clouds overlaid with reflectivity data.

Once background detections have been rejected, 3D estimation using photon arrival times, $\{t(x, y)\}$ becomes tractable. Our final processing step computes the regularized maximum-likelihood 3D estimate by maximizing the product of data likelihoods (Eq. 2), over the uncensored spatial locations, combined with a DWT-based penalty function that exploits spatial correlations present in a scene's 3D structure (16).

After radiometric calibration, we tested first-photon imaging on scenes with both geometric and reflectivity complexity. One object investigated was a life-sized mannequin with white polystyrene head and torso donned with a black cotton shirt imprinted with white text. The approximate dimensions of the head were 20 cm by 16.5 cm by 24 cm, and those of the torso were 102 cm by 42 cm by 29 cm. The mannequin was placed at a range of 1.5 m from the imaging setup. By using the first-photon data, we estimated the object reflectivity and 3D spatial form as described above. For a 1-megapixel reflectivity and 3D reconstruction, our data acquisition time was about 20 min, and the computations took less than 3 min on a desktop computer. A standard graphics package was then used to visualize the object's 3D profile, overlaid with reflectivity data, after each processing step (Fig. 2, D to L, and movie S1).

Our first-photon imager recovers a great deal of object detail. Figures 2, D to F, and 3 show recovery of reflectivity information—including text, facial features, surface reflectivity variations, and specular highlights—that are heavily obscured in pointwise maximum-likelihood estimates. As shown in Fig. 2, G to I, background-detection censoring affords substantial improvement in range estimation, so that the reconstructed 3D form reveals fine structural features, such as the nose, eyes, and lips (Fig. 2, J to L).

To quantify the accuracy of our approach, we compared the 3D reconstruction of the mannequin head with a 3D image captured with our imaging setup operating as a direct-detection LIDAR. For this reference capture, background light was first reduced to negligible levels, after which ~ 1000 photon detections were recorded at each spatial location. Pointwise 3D estimates were then obtained from a photon-counting histogram. This data-intensive baseline technique allows sub-mm-accuracy 3D reconstruction for our $T_p = 226$ ps value (20).

Figure 4 shows superimposed facial profiles from the two methods. Both 3D forms were measured by using the same imaging setup, obviating the need for registration or scaling. The RMS error of our computational imager was slightly lower than 3.5 mm, with higher values near the edge of the mannequin and around the sides of the nose and the face. These locations have surface normals that are nearly perpendicular to the line of sight, which dramatically reduces their back-reflected signal strength relative to background light. Consequently, they incur more anomalous detections than do the rest of the pixels. Although

our method censors range anomalies near edges, it estimates the missing ranges by using spatial correlations, leading to loss of subtle range details at these edges.

The range resolution of our computational imager was measured with a test target and found to be about 4 mm (figs. S2 to S5). This resolution is 8.5 times smaller than $cT_p/2 = 34$ mm (our

setup's RMS error for pointwise first-photon range estimation in the absence of background light) and 765 times smaller than $(c/2)\sqrt{(T_p^2 + T_i^2)/12}/2 = 3.06$ m (our setup's RMS error for pointwise first-photon range estimation in the presence of background light) (16). Moreover, LIDAR-mode pointwise range estimation from a photon-counting histogram required at least 115 photons per pixel

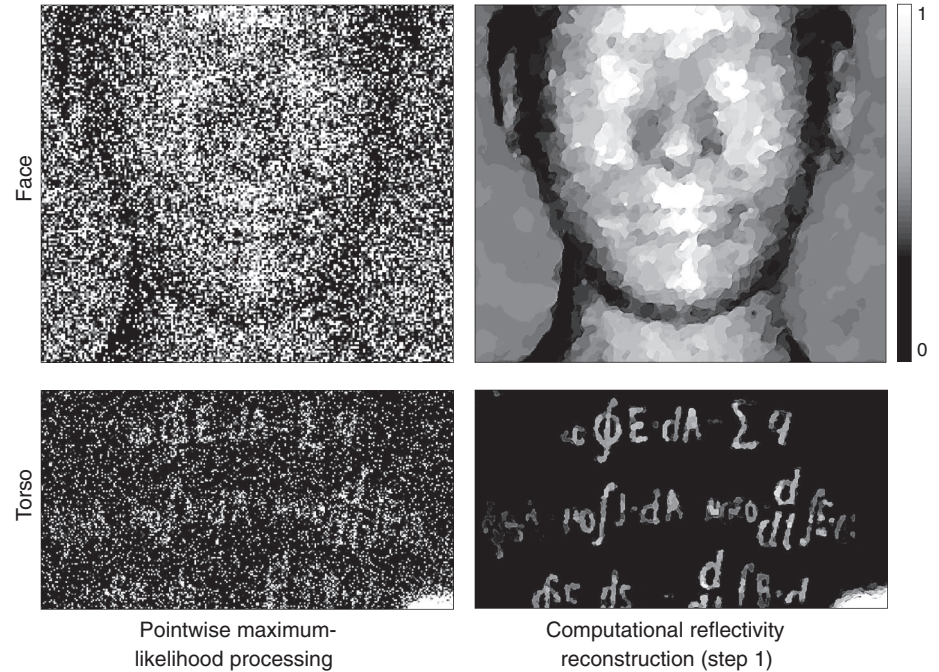


Fig. 3. Reflectivity reconstruction from first-photon detections. The scale quantifies reflectivity relative to that of a high-reflectivity calibration point, $\alpha(x_{\text{ref}}, y_{\text{ref}})$, on a 0 to 1 scale.

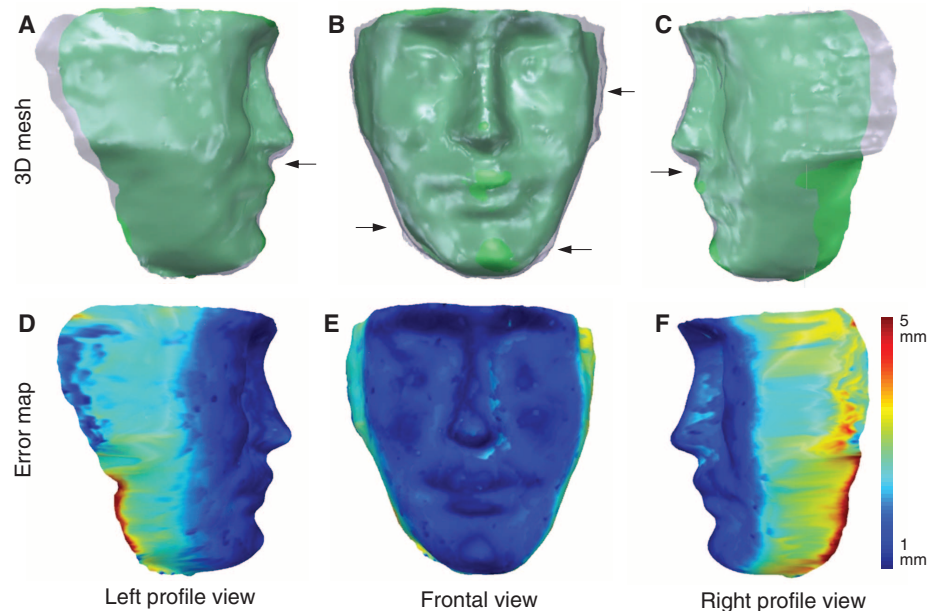


Fig. 4. Comparison between computational first-photon 3D imager and LIDAR system. Rendered views of the facial surfaces reconstructed with computational imaging (gray) and 3D LIDAR (green) are shown in frontal (B) and lateral (A) and (C) profiles with arrows indicating the locations of significant range discrepancies. Color-coded absolute pointwise differences between the two surfaces, overlain on the LIDAR reconstruction, are shown in (D) to (F).

to achieve a 4-mm RMS error under identical imaging conditions.

Reflectivity measurements with a test pattern showed that our method provided at least 4 bits of information; that is, 16 linear gray-scale levels could be distinguished (figs. S6 to S9). We also tested the repeatability of our method by collecting 500 independent first-photon data trials for the mannequin. We found our first-photon imager to consistently demonstrate qualitative and quantitative improvement over pointwise processing (movie S2). Its performance was also reproducible with other real-world scenes composed of multiple distinct objects at different ranges (figs. S10 to S12).

For completeness, we compared our imager with image-denoising techniques. One such method median filters the pointwise estimates (Fig. 2, A to C). Its reduction of noise resulting from anomalous detections comes at the expense of image oversmoothing, which leads to loss of perceptual information contained in edges, reflectivity, and structural variations. State-of-the-art denoising algorithms, like BM3D (21), exploit spatial correlations to mitigate high levels of noise, but they fail to match the performance of our imager because first-photon detection statistics differ from the conventional noise models that such algorithms presume (figs. S13 and S14).

Our computational first-photon imaging technique achieves its high-quality performance by using spatial correlations to suppress Poisson noise in reflectivity images and censor range anomalies from arrival-time data. It extracts more information from the collection of single detections than state-of-the-art active imagers would. Thus, it allows laser power to be reduced with-

out sacrificing image quality, something that can be crucial for biological applications, such as fluorescence-lifetime imaging (22, 23). It also enables remote sensing at longer standoff distances with power-limited transmitters and could be combined with techniques for detecting multiple depths per pixel (24). The system we have demonstrated can be improved with better background-light suppression (25), range gating (26), and advances in single-photon detector technology (27, 28).

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Supplementary Materials

www.sciencemag.org/content/343/6166/58/suppl/DC1

Materials and Methods

Figs. S1 to S14

Table S1

References (29–31)

Movies S1 and S2

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Direct Stereospecific Synthesis of Unprotected N-H and N-Me Aziridines from Olefins

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Despite the prevalence of the N-H aziridine motif in bioactive natural products and the clear advantages of this unprotected parent structure over N-protected derivatives as a synthetic building block, no practical methods have emerged for direct synthesis of this compound class from unfunctionalized olefins. Here, we present a mild, versatile method for the direct stereospecific conversion of structurally diverse mono-, di-, tri-, and tetrasubstituted olefins to N-H aziridines using *O*-(2,4-dinitrophenyl)hydroxylamine (DPH) via homogeneous rhodium catalysis with no external oxidants. This method is operationally simple (i.e., one-pot), scalable, and fast at ambient temperature, furnishing N-H aziridines in good-to-excellent yields. Likewise, N-alkyl aziridines are prepared from N-alkylated DPH derivatives. Quantum-mechanical calculations suggest a plausible Rh-nitrene pathway.

Aziridines, the triangular, comparably highly strained nitrogen analogs of epoxides, are important synthetic intermediates (i.e., building blocks) en route to structurally complex

molecules because of their versatility in myriad regio- and stereoselective transformations (ring openings and expansions, as well as rearrangements) (1–6). The aziridine structural motif,

predominantly N-H and to a lesser extent N-alkyl, also appears in biologically active natural products (e.g., azinomycins and mitomycins) (7–9). As a result, the synthesis and chemistry of aziridines have been the subject of intense research during the past 25 years, resulting in multiple aziridination methods (10–23). Most of these methods rely either on the transfer of substituted nitrenes, which are generated by using strong external oxidants, to the C=C bond of olefins or the transfer of substituted carbenes to the C=N bond of imines. Normally, the result is an aziridine bearing a strongly electron-withdrawing N-protecting group (e.g., Ts: *para*-toluenesulfonyl; Ns: *para*-nitrophenylsulfonyl); removal of these *N*-sulfonyl protecting groups is problematic as it often results in the undesired

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opening of the aziridine ring. In addition, the high reactivity of N-protected nitrenes might give rise to nonproductive allylic C-H amination products, as well as the loss of stereospecificity. Clearly, the direct synthesis of N-H (i.e., N-unprotected) and N-alkyl aziridines would alleviate the above problems. However, a practical, functional group-tolerant and environmentally benign direct preparation of N-H aziridines from structurally diverse olefins has so far eluded synthetic chemists (24–31). Here, we report an operationally simple, inherently safe, chemoselective and stereospecific conversion of a wide range of olefins to the corresponding N-H or N-Me aziridines via a rhodium-catalyzed pathway free of external oxidants.

Recently, we developed a metal-free protocol for primary amination of arylboronic acids using only *O*-(2,4-dinitrophenyl)hydroxylamine (DPH, **1a**, Fig. 1) as the stoichiometric aminating agent (32). The transformation proceeds under neutral or basic conditions and can be conducted on a multigram scale to provide structurally diverse primary arylamines. The versatility and robustness of **1a** prompted us to explore other uses of this aminating agent, specifically for the direct functionalization of readily available and inexpensive olefins. Our investigations began by subjecting 1:1.5 mixtures of *cis*-methyl oleate (**7**)/**1a**, as well as styrenes (**3a** and **3b**)/**1a**, to a vigorous screening with various transition metal complexes (tables S1 and S2). This initial screen identified $\text{Rh}_2(\text{OAc})_4$ as a promising catalyst for *vic*-amino-oxyarylation of olefins. Further evaluation of dimeric rhodium dicarboxylate complexes (table S3) revealed that just 1 mol % loading of Du Bois' catalyst (33–36) (**2**, Fig. 1) in acetonitrile (MeCN) leads to amino-oxyarylated styrenes **4a** and **4b** at room temperature in 56% and 75% isolated yields, respectively. These promising results prompted us to conduct a thorough solvent screen.

In methanol, we observed the incorporation of the MeO group at the benzylic position (**5**) in addition to the amino-oxyarylated product **4b**; these compounds were isolated in a combined yield of 78%. At this juncture, we reasoned that a highly polar, hydroxylic, and nonnucleophilic solvent such as 2,2,2-trifluoroethanol ($\text{CF}_3\text{CH}_2\text{OH}$, TFE) would completely avoid the incorporation of solvent into the products. Indeed, **3b** was cleanly amino-oxyarylated in TFE, and **4b** was isolated in 66% yield. It was unclear whether the transformation **3b**→**4b** involved the opening of a highly reactive aziridine (**6**) or an alternative process. Surprisingly, when **7** was reacted in trifluoroethanol as solvent, *cis*-N-H aziridine **8** was isolated in excellent yield (83%) instead of the expected amino-oxyarylated product. The transformation proceeded with complete stereospecificity as no traces of the *trans*-N-H aziridine were detected by ^1H - and ^{13}C -nuclear magnetic resonance (^{13}C -NMR) analysis ($\leq 2\%$ sensitivity).

Encouraged by this unexpected result, we initiated a systematic study using representative aliphatic olefins with a wide range of substitution patterns and functionalities (Fig. 2). Terminal ali-

phatic olefin substrates (entries 1 to 3, Fig. 2) either did not react or reacted sluggishly (i.e., days) when 1 mol % of catalyst **2** was used; however, increasing the catalyst loading to 5 mol % led to rapid conversion at room temperature to the corresponding N-H aziridines (**10a** to **10c**). We empirically found that in some of the reactions (i.e., entries 4, 5, 7, 9, 11, 14, and 20), addition of the catalyst in several 1 mol % portions minimized decomposition of both the catalyst and aminating agent and invariably led to higher isolated yield of product. Notably, the N-H aziridination took place efficiently in the presence of a labile terminal epoxide (**10c**), as well as an unprotected primary alcohol (**10a**); these functionalities typically interfere with currently used aziridination protocols. In case of the transformation **9c**→**10c**, only the product was detected in the crude reaction mixture by NMR analysis. In the presence of 1 mol % of catalyst **2**, both *cis*- and *trans*-1,2-disubstituted aliphatic olefins (entries 4 to 10, Fig. 2) underwent smooth and stereospecific N-H aziridination at room temperature as established by ^{13}C -NMR analysis ($\leq 2\%$ sensitivity). The presence of an unprotected secondary alcohol in substrate **9i** (entry 9) did not influence the stereochemical outcome of the N-H aziridination and **10i** was isolated as a 1:1 mixture of diastereomers.

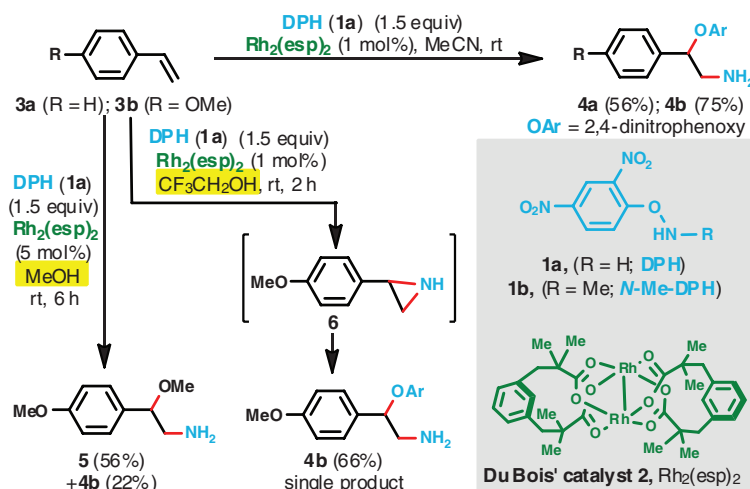
Benzoyloxy and acetyloxy *cis*-olefins **9k** and **9m** (entries 11 and 14), when exposed to 1 mol % of catalyst **2** and 1.2 equivalents of aminating agent **1a** at 50°C, were smoothly aziridinated followed by an in situ aziridine ring-opening (via transacylation) to yield the corresponding *trans*-

2,3-disubstituted furans **10kk** and **10mm** in 84% and 61% yields, respectively. By contrast, when olefin **9k** was exposed to 5 mol % loading of catalyst **2** and 1.2 equivalents of **1a** at 25°C, the expected N-H aziridine **10k** (entry 12) was formed in just 2 hours and isolated in 69% yield. As anticipated, when the rate of N-H aziridination is slow and elevated temperatures are used, secondary processes (i.e., intramolecular annulation) that consume the initially formed N-H aziridines can dominate. Apparently, a fivefold increase in catalyst loading increased the rate of N-H aziridination sufficiently that it could take place rapidly at ambient temperature.

Cyclohexene **9n** (entry 15) was aziridinated at room temperature to afford cyclic N-H aziridine **10n**; no traces of allylic C-H amination (i.e., 1-amino-2-cyclohexene) could be detected by ^1H -NMR analysis ($\leq 2\%$ sensitivity), in sharp contrast with other metal nitrene-based aziridination methods (37). Geraniol (**9o**, entry 16) and geranyl acetate (**9q**, entry 18), which incorporate two trisubstituted C=C double bonds, were N-H aziridinated regioselectively, favoring the double bond at the $\Delta^{6,7}$ -position over the $\Delta^{2,3}$ -position in both cases.

The shift of the regioisomeric ratio from 1:5 in **10o** to 1:14 in **10q** suggests a subtle directing effect of the free allylic alcohol and/or an inductive deactivation by the acetate; perhaps the extent of H-bonding in the solvent also plays a role. Entry 17 stands as a testament to the extraordinarily mild reaction conditions as trisubstituted olefin **9p**, which possesses a highly sensitive epoxy

A Initial screening of catalytic systems:



B Formation of an N-H aziridine:

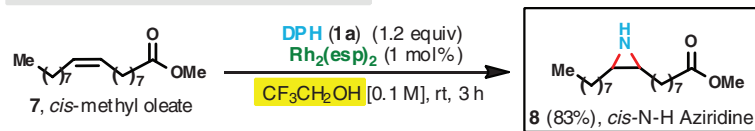


Fig. 1. Exploration of DPH (1a**) as a versatile aminating agent.** (A) $\text{Rh}_2(\text{esp})_2$ is an effective catalyst for olefin difunctionalization. (B) In 2,2,2-trifluoroethanol (TFE or $\text{CF}_3\text{CH}_2\text{OH}$), **7** undergoes direct aziridination to N-H aziridine **8** in excellent isolated yield.

alcohol, was aziridinated rapidly and efficiently to epoxy N-H aziridine **10p** in excellent yield. The transformation **9q**→**10q** (entry 18) could be readily scaled up (6 mmol) with minimal erosion of the isolated yield to provide gram quantities of **10q**. N-H aziridination of limonene **9r** (entry 19) favored the trisubstituted ring double bond with 9:1 regioselectivity; however, the chiral center had no evident influence on the diastereoselectivity (1:1 drdr, diastereomeric ratio). In contrast with the lack of stereoselectivity in **9i**, cholesterol **9s** (entry 20) exclusively yielded the β-N-H aziridine **10s** in 71% yield; this unexpected stereochemical out-

come, confirmed by single-crystal x-ray analysis of **10ss** (a crystalline derivative of **10s**), suggests a directing effect by the adjacent C(3)-β-alcohol not observed in conformationally more mobile acyclic molecules such as **9i**. The success with cholesterol and other natural products (**7**, **9h**, **9i**, **9o** and **9r**; Figs. 1 and 2) highlights the prospective utility of this method in the straightforward elaboration of molecules of biomedical interest (e.g., for ¹⁵N-labeling studies).

Next, we turned our attention to the direct N-H aziridination of di-, tri- and tetrasubstituted styrenes and stilbene (entries 21 to 28, Fig. 3A).

In general, styrenes were more reactive than aliphatic olefins, and often lower temperatures (−10° to 25°C) were adequate. Conspicuously, *cis*-β-methyl styrene **11d** furnished the corresponding *cis*-2-Ph-3-Me N-H aziridine (**12d**, entry 24) without isomerization. Similarly, *trans*-β-methyl styrene **11c** readily furnished *trans*-2-Ph-3-Me N-H aziridine (**12c**, entry 23) even on a 1- to 8-mmol scale. The N-H aziridine derived from 2-Me indene (**12h**, entry 28) was not isolated owing to its high reactivity, but instead reduced in situ to amine **12hh**. Evaluation of the effect of catalyst loading on the reaction **11f**→**12f** (entry 26) revealed that the

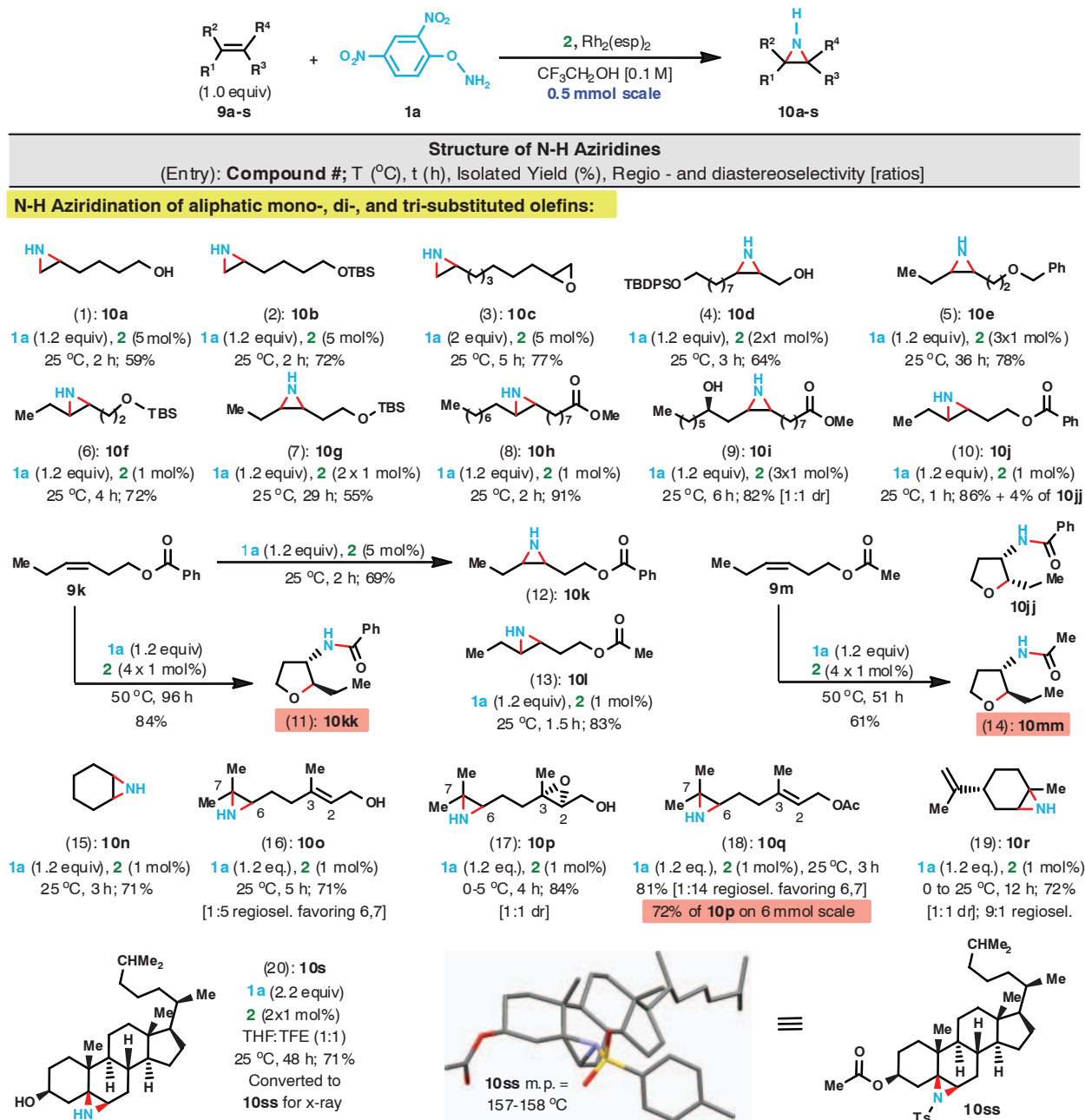


Fig. 2. Direct and stereospecific N-H aziridination of olefins. Reactions were conducted at 0.1 M using 2,2,2-trifluoroethanol as solvent and at 0.5-mmol scale unless otherwise indicated. To obtain crystalline material, **10s** was *O*-acetylated and *N*-tosylated (Ts, *para*-toluenesulfonyl) to afford derivative **10ss**. (TBS, *tert*-butyl-dimethylsilyl; TBDPS, *tert*-butyl-diphenylsilyl)

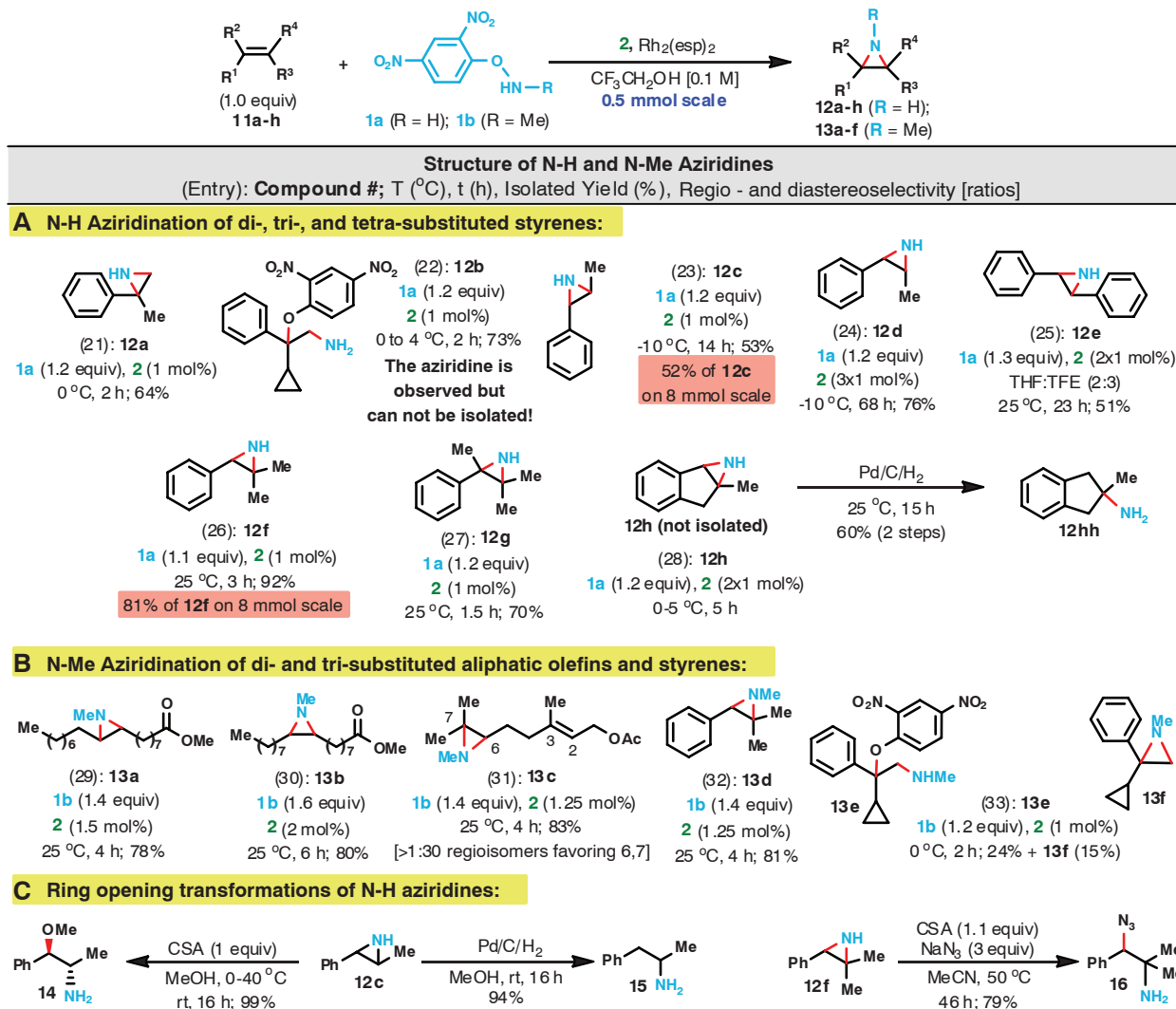


Fig. 3. Direct and stereospecific N-H and N-Me aziridination of olefins. Reactions were conducted at 0.1 M using 2,2,2-trifluoroethanol as solvent and at 0.5-mmol scale unless otherwise indicated. CSA, camphorsulfonic acid.

lowest practical loading of catalyst **2**, without decreasing the isolated yield or drastically increasing the reaction time, was 0.5 mol %. This low catalyst loading renders the process economical and environmentally friendly. A further fivefold reduction in catalyst loading (from 0.5 to 0.1 mol %) resulted in a 25-fold increase in reaction time and a 30% drop in the isolated yield of **12f**. Tetra-substituted olefin **11g** (entry 27) was easily N-H aziridinated at room temperature; **12g** was isolated in 70% yield. The attempted direct N-H aziridination of 1-Ph-1-cyclopropylethene (**11b**) yielded only amino-oxyarylated product **12b**; the complete lack of cyclopropane ring-opening products corroborate an aziridination pathway that does not involve long-lived radical or carbocation intermediates (see more detailed discussion of the mechanism in the computation section and in Fig. 4).

The practicality and broad scope of the preceding direct and stereospecific N-H aziridination of olefins (Fig. 2 and Fig. 3A) prompted an investigation of direct N-Me aziridination. Toward

this end, several di- and trisubstituted aliphatic olefin and styrene substrates (entries 29 to 33, Fig. 3B) were examined in the presence of **1b** as the stoichiometric aminating agent and 1 to 2 mol % of catalyst **2**. The N-Me aziridination of olefins also proceeded stereospecifically (entries 29 and 30) and, in the case of geraniol acetate **9q**, the regioselectivity increased from 1:14 (in **10q**) to >1:30 (in **13c**), favoring the $\Delta^{6,7}$ -olefin in both cases.

Two of the N-H aziridine products (**12c** and **12f**) were subjected to ring-opening transformations (Fig. 3C). Upon catalytic hydrogenation, aziridine **12c** afforded a 94% yield of amphetamine **15**, the active pharmaceutical ingredient in Adderall, an approved medication for attention deficit hyperactivity disorder, as well as narcolepsy, that is marketed as a mixture of enantiomers. Under acidic conditions, at slightly elevated temperature (40 °C) in MeOH, **12c** was converted to *O*-Me-norephedrine **14** with complete regioselectivity and in nearly quantitative yield. Likewise,

the ring-opening of trisubstituted N-H aziridine **12f** with sodium azide furnished azidoamine **16** in 79% yield. These transformations by example illustrate how readily a nitrogen atom can be introduced into molecules.

We also examined prospective reaction mechanisms using quantum mechanical density-functional theory calculations (Fig. 4). Our (U)M06 calculations were carried out in Gaussian 09 (38) using a polarizable conductor continuum solvent model for trifluoroethanol. Details of calculated transition states and intermediates are given in the supplementary materials.

We first examined plausible rhodium nitrene pathways. Generation of a rhodium nitrene intermediate is possible if the amino group of **1a** coordinates to $\text{Rh}_2(\text{esp})_2$ followed by loss of dinitrophenol (pathway A, Fig. 4). Calculations suggest that the triplet-spin state of the nitrene ($^3\mathbf{17}$) is more than 8 kcal/mol lower in energy than the open-shell singlet, and reaction pathways identified on the triplet-spin energy surface were

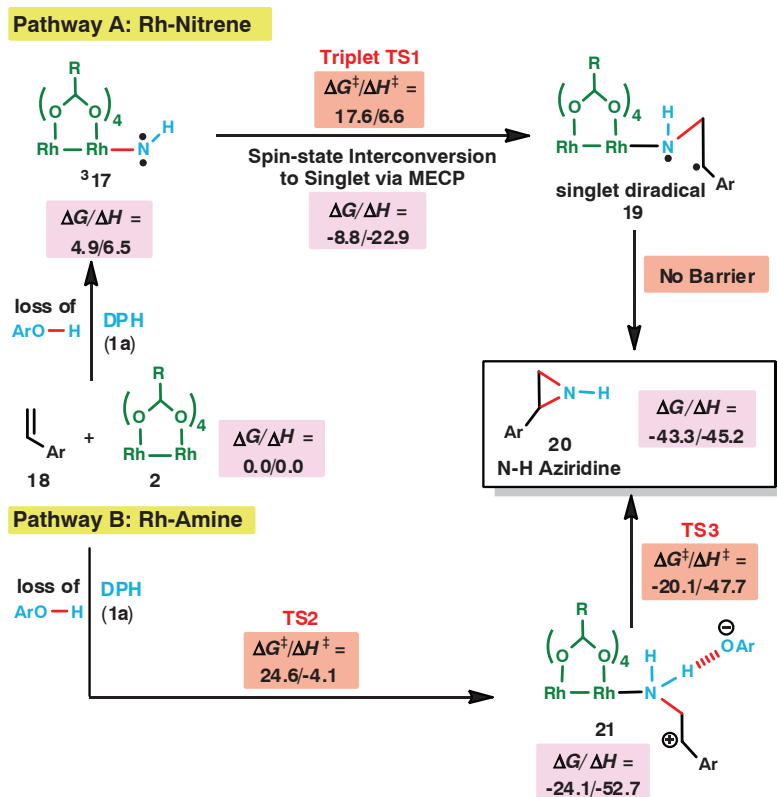


Fig. 4. Selected DFT-examined pathways for N-H aziridination of styrene in 2,2,2-trifluoroethanol solvent. R, esp ligands. Energies in kcal/mol. MECP, minimum energy crossing point.

found to be lower in energy than reaction pathways on the singlet-spin energy surface (39, 40). Because the $Rh_2(esp)_2$ catalyst and aziridine product have singlet-spin ground states, the reaction pathway must involve spin interconversion. The mechanism outlined in Fig. 4 provides a route for stereospecific aziridination if $^3\mathbf{17}$ reacts with alkenes by forming the first C–N bond via triplet transition state **TS1** followed by spin interconversion along the pathway to diradical intermediate **19** or fast spin interconversion at the diradical intermediate (41). After spin interconversion, the second C–N bond is formed by the coupling of singlet-paired electrons without a barrier and leads directly to aziridine **20**.

As alternatives to nitrene pathways, we also explored polar mechanisms involving Rh-amine and Rh-alkene coordination modes (see supplementary materials). One of several possible polar mechanisms is outlined as pathway B in Fig. 4. This pathway is akin to the mechanism proposed for amination of aryl boronic acids with **1a** (32). Although this mechanism may account for amino-oxyarylated products (e.g., **4a** and **4b**) observed under some experimental conditions, the calculated barrier for this mechanism, as well as alternative polar mechanisms, is higher in energy than the nitrene mechanism presented in pathway A.

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Supplementary Materials

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Materials and Methods
Figs. S1 to S5
Tables S1 to S8
References (42–69)

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Tunable Electrical Conductivity in Metal-Organic Framework Thin-Film Devices

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We report a strategy for realizing tunable electrical conductivity in metal-organic frameworks (MOFs) in which the nanopores are infiltrated with redox-active, conjugated guest molecules. This approach is demonstrated using thin-film devices of the MOF $\text{Cu}_3(\text{BTC})_2$ (also known as HKUST-1; BTC, benzene-1,3,5-tricarboxylic acid) infiltrated with the molecule 7,7,8,8-tetracyanoquinodimethane (TCNQ). Tunable, air-stable electrical conductivity over six orders of magnitude is achieved, with values as high as 7 siemens per meter. Spectroscopic data and first-principles modeling suggest that the conductivity arises from TCNQ guest molecules bridging the binuclear copper paddlewheels in the framework, leading to strong electronic coupling between the dimeric Cu subunits. These ohmically conducting porous MOFs could have applications in conformal electronic devices, reconfigurable electronics, and sensors.

Metal-organic frameworks [MOFs (*1–3*)] are crystalline materials with a nanoporous, supramolecular structure consisting of metal ions connected by multitopic organic ligands. These materials are typically poor electrical conductors because of the insulating character of the organic ligands and poor overlap between their π orbitals and the d orbitals of the metal ions. Combining the crystalline order of MOFs with an ability to conduct electrical charge has the potential to create a new class of materials that would enable applications such as conformal electronic devices, reconfigurable electronics, and sensors. Although strategies to engineer electrically conducting MOFs have been proposed (e.g., the use of second- or third-row transition metals, redox-active linkers, and hetero-bimetallic structures), few of these approaches have been realized (*4*). Until recently, only one example of an intrinsically conducting framework with permanent porosity was known: a p-type semiconducting MOF in which conductivity occurs via a redox mechanism (*5*). Very recently, Gándara *et al.* described a series of metal triazolate MOFs, one of which exhibits ohmic conductivity (*6*). The mechanism of conductivity in that case is not known, but it appears to be highly specific to the presence of Fe(II) in the structure, as MOFs in this series with the same structure but different divalent metals are not conducting.

An alternative approach is to use the MOF pores themselves as a venue for modulating the electrical transport properties. We reasoned that infiltrating MOFs having open metal sites with

molecules capable of charge transfer that can coordinate to these sites would create a mechanism for carrier mobility. Binuclear Cu(II) paddlewheel MOF structures such as $\text{Cu}_3(\text{BTC})_2$ (*7*) (also known as HKUST-1; BTC, benzene-1,3,5-tricarboxylic acid) are attractive candidates for testing this strategy. The coordination positions located on opposite ends of the Cu(II) axis of the paddlewheel—which are occupied by solvent (often water) in the as-synthesized material—can be exchanged with redox-active molecules such as TCNQ. More-

over, electron paramagnetic resonance (EPR) spectra suggest a somewhat delocalized electronic structure enabled by additional spin exchange among the copper dimers, which results from the carboxylates interconnecting the paddlewheel subunits (*8*).

Here, we describe the realization of this strategy using a thin-film device comprising $\text{Cu}_3(\text{BTC})_2$ (*7*) grown on electrodes and demonstrate control of ohmic electrical conductivity over six orders of magnitude. Silicon wafers covered with 100 nm of SiO_2 were prepatterned with Pt pads 100 nm thick (dimensions 800 μm by 400 μm) and gaps of 100 μm , 150 μm , and 200 μm . $\text{Cu}_3(\text{BTC})_2$ films with nominal thickness of 100 nm were grown on the wafers from the liquid phase, as described (*9*). Grazing incidence x-ray diffraction (XRD) measurements and scanning electron microscopy (SEM) imaging (Fig. 1, B and C) indicate a polycrystalline $\text{Cu}_3(\text{BTC})_2 \cdot x\text{H}_2\text{O}$ film with preferred orientation along the (111) direction (*10*). Current-voltage (*I-V*) characteristics obtained on as-grown thin-film devices in air (Fig. 2A) exhibited very low conductivity ($\sim 10^{-6}$ S/m), consistent with the expected insulating nature of $\text{Cu}_3(\text{BTC})_2$.

The as-grown films were infiltrated with TCNQ by first heating in vacuum at 190°C for 30 min to remove coordinated water molecules, then immediately transferring them to a saturated TCNQ/ CH_2Cl_2 solution. *I-V* curves for four such devices after 72 hours of exposure to the TCNQ solution are shown in Fig. 2A (*11*). The infiltration led to marked increases in current, with a linear *I-V* curve and conductivity of 7 S/m, six

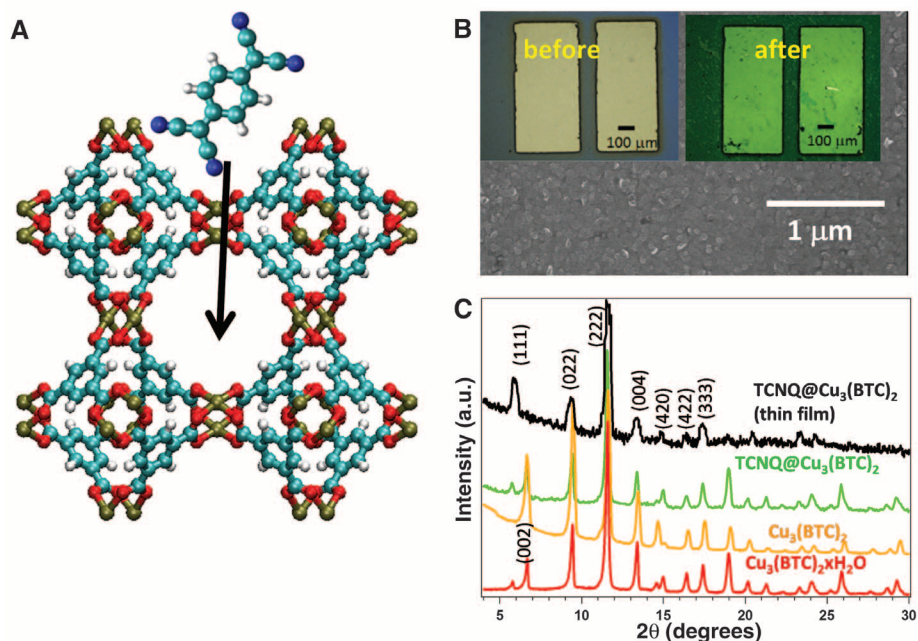


Fig. 1. Fabrication of conductive MOF thin-film devices and structural characterization. (A) TCNQ molecule shown above a $\text{Cu}_3(\text{BTC})_2$ MOF; arrow points into the pore. White, hydrogen; blue, nitrogen; cyan, carbon; red, oxygen; light brown, copper. (B) SEM image of MOF-coated device; insets are optical images of devices before and after TCNQ infiltration. (C) XRD data for powders and grazing incidence XRD for a thin film.

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orders of magnitude greater than that of the un-infiltrated device. Measurements as a function of channel length (Fig. 2B) revealed a monotonic increase in resistance with increasing electrode separation. The TCNQ-infiltrated devices were stable in ambient atmosphere up to at least 40 days (Fig. 2C). The conductivity increased with temperature (Fig. 2, D and E), following a thermally activated relation $\sigma \sim \exp(E_a/T)$ with a low activation energy $E_a = 41 \pm 1$ meV, similar to values reported for high-mobility organic polymeric semiconductors such as poly-3-hexylthiophene (P3HT) (12).

In contrast to nonporous conducting coordination polymers such as CuTCNQ (12–15), we could tune the device conductivity by changing the TCNQ exposure time. As seen in Fig. 2F, conductivity variation over several orders of magnitude was achievable. Furthermore, the increase in conductivity could be described by classic percolation theory (black solid line) (16), which suggests that TCNQ forms localized conducting regions rather than acting as a dopant.

Chemical and physical characterization of MOF films and powders exposed to TCNQ confirmed that TCNQ resides in the MOF pores without altering the MOF crystal structure. Powder XRD patterns of as-synthesized $\text{Cu}_3(\text{BTC})_2 \cdot x\text{H}_2\text{O}$, $\text{Cu}_3(\text{BTC})_2$ (activated), and $\text{Cu}_3(\text{BTC})_2$ infiltrated with TCNQ [hereafter $\text{TCNQ}@ \text{Cu}_3(\text{BTC})_2$] showed that the MOF crystalline structure (face-centered cubic) was unaffected by the infiltration process (Fig. 1C and fig. S2) (7). Rietveld refinement yielded lattice parameters of 2.617 ± 0.001 nm and 2.635 ± 0.001 nm for $\text{Cu}_3(\text{BTC})_2$ and $\text{TCNQ}@ \text{Cu}_3(\text{BTC})_2$ powders, respectively. The peak at $2\theta = 5.759^\circ$ seen in the patterns of both $\text{Cu}_3(\text{BTC})_2 \cdot x\text{H}_2\text{O}$ and $\text{TCNQ}@ \text{Cu}_3(\text{BTC})_2$ (but absent from that of activated MOF) is diagnostic for guest molecule binding to the open metal sites in the large pores,

and indicates long-range order (17). The surface area of the activated $\text{Cu}_3(\text{BTC})_2$ powder, obtained from N_2 adsorption isotherms using the Brunauer-Emmett-Teller (BET) method, is 1844 ± 4 $\text{m}^2 \text{g}^{-1}$. This value is typical of high-quality $\text{Cu}_3(\text{BTC})_2$ $Fm\bar{3}m$ material with little or no pore collapse or residual reactant (18). After infiltration and drying in air, the $\text{TCNQ}@ \text{Cu}_3(\text{BTC})_2$ material displayed a BET surface area of 214 ± 0.5 $\text{m}^2 \text{g}^{-1}$, suggesting high TCNQ loading. This result was confirmed by elemental analysis indicating a $\text{Cu}_3(\text{BTC})_2/\text{TCNQ}$ ratio of 2 on the basis of carbon, nitrogen, and hydrogen content. This corresponds to about eight TCNQ molecules per unit cell, or one TCNQ molecule per MOF pore. The presence of nitrogen in the $\text{TCNQ}@ \text{Cu}_3(\text{BTC})_2$ films was further corroborated by x-ray photoelectron spectroscopy (XPS) (fig. S3). Furthermore, visual examination of the powdered MOFs revealed the expected turquoise-blue color for the as-synthesized material and the violet-blue hue for the activated (dehydrated) MOF (fig. S1). Upon exposure to TCNQ, the color of the crystals changed to teal, indicating a perturbation of the MOF electronic structure. The color of $\text{TCNQ}@ \text{Cu}_3(\text{BTC})_2$ did not change upon exposure to air (fig. S1), which suggests that TCNQ is not displaced by atmospheric water vapor; XPS also showed a substantially lower oxygen concentration in the infiltrated specimen (fig. S3) consistent with reduced water occupation of the pores. In contrast, the color of the activated MOF before TCNQ infiltration reverted almost instantly to that of the as-synthesized (hydrated) material when exposed to atmospheric moisture.

The realization of these hybrid electronic materials raised questions concerning the nature of the TCNQ-MOF interaction and the mechanism of charge transport. We probed the TCNQ-MOF interaction in several ways. Ultraviolet-visible

(UV-vis) spectra were collected from films of the un-infiltrated $\text{Cu}_3(\text{BTC})_2 \cdot x\text{H}_2\text{O}$, $\text{TCNQ}@ \text{Cu}_3(\text{BTC})_2$, and TCNQ in dilute solution. The absorption spectrum of the $\text{TCNQ}@ \text{Cu}_3(\text{BTC})_2$ film (Fig. 3A) exhibited the expected MOF peak at 340 nm, a peak at 410 nm associated with neutral TCNQ (18), and broad new absorption bands centered at ~ 700 nm and ~ 850 nm that were absent in both $\text{Cu}_3(\text{BTC})_2 \cdot x\text{H}_2\text{O}$ and TCNQ in CH_2Cl_2 . These additional bands are well-known signatures for charge transfer (13, 19). Note that reacting either copper acetate or copper sulfate with TCNQ in methanol generated no precipitates or new absorption bands, indicating that confinement in the MOF pore is essential to the formation of a charge transfer complex between Cu(II) and TCNQ.

TCNQ complexes have been characterized extensively by vibrational spectroscopies, where the frequencies of C=C and C=N stretching modes are particularly sensitive to the extent of charge transfer (13, 20, 21). Raman spectra of $\text{TCNQ}@ \text{Cu}_3(\text{BTC})_2$ (Fig. 3B) indicate that the TCNQ C=C stretching frequency shifted from 1456 cm^{-1} to 1437 cm^{-1} and new peaks appeared at 1352 cm^{-1} and 1296 cm^{-1} —a strong indication that TCNQ interacts with the available coordination sites on the Cu^{2+} ions in the framework. A shift of 19 cm^{-1} for the C=C wing stretching mode suggests a partial charge transfer of $\sim 0.3e^-$ between the framework and TCNQ (20). The nitrile stretch at 2229 cm^{-1} is split into two peaks at 2226 cm^{-1} and 2213 cm^{-1} (Fig. 3B, inset) indicating two non-equivalent C=N bonding environments, in close agreement with our calculated vibration spectra (11). Infrared spectra (Fig. 3C) also show that the C=N stretch of TCNQ is affected by adsorption into the framework, with a shift from 2223 cm^{-1} to 2204 cm^{-1} accompanied by substantial peak broadening. According to Chappell *et al.* (21), this shift corresponds to a charge transfer of $\sim 0.4e^-$ between

Fig. 2. Electronic transport characteristics of MOF thin-film devices.

(A) *I*-*V* curves before (red) and after infiltration with TCNQ (green), F4-TCNQ (gold), or H4-TCNQ (purple). (B) Channel-length dependence of conductivity for TCNQ-infiltrated devices; error bars denote SD. (C) Stability of conductivity over time for several devices. (D) *I*-*V* curve temperature dependence. (E) Arrhenius plot of the conductivity. (F) Conductivity versus exposure time for several devices. The black line is a fit to percolation theory, $\sigma = 4 \times 10^{-6}(t - 0.5)^2$, where t is exposure time.

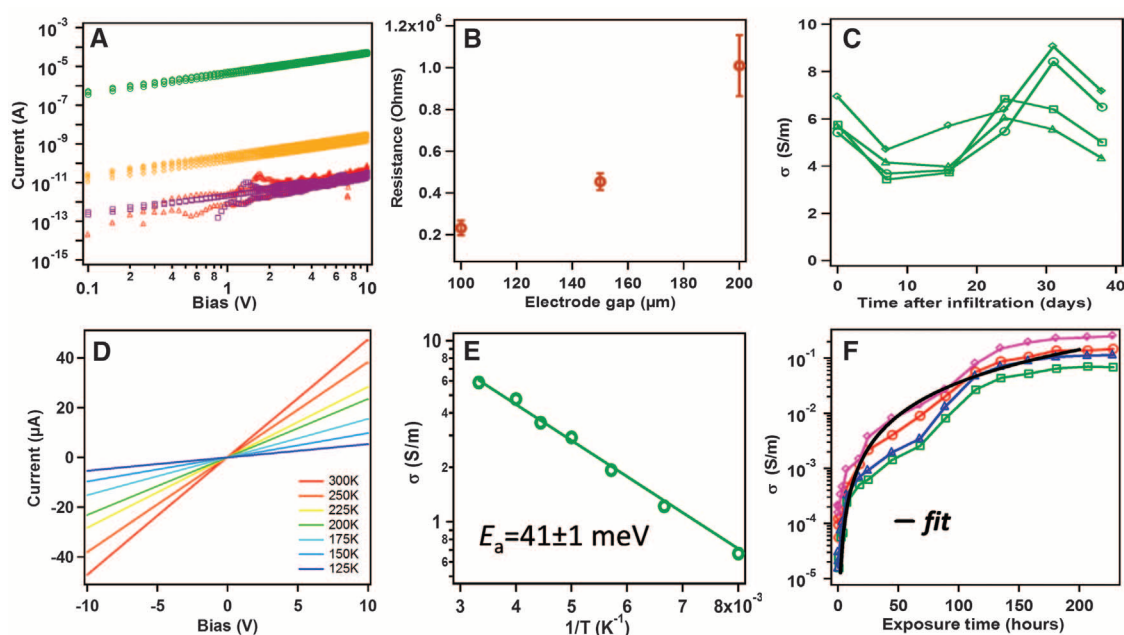
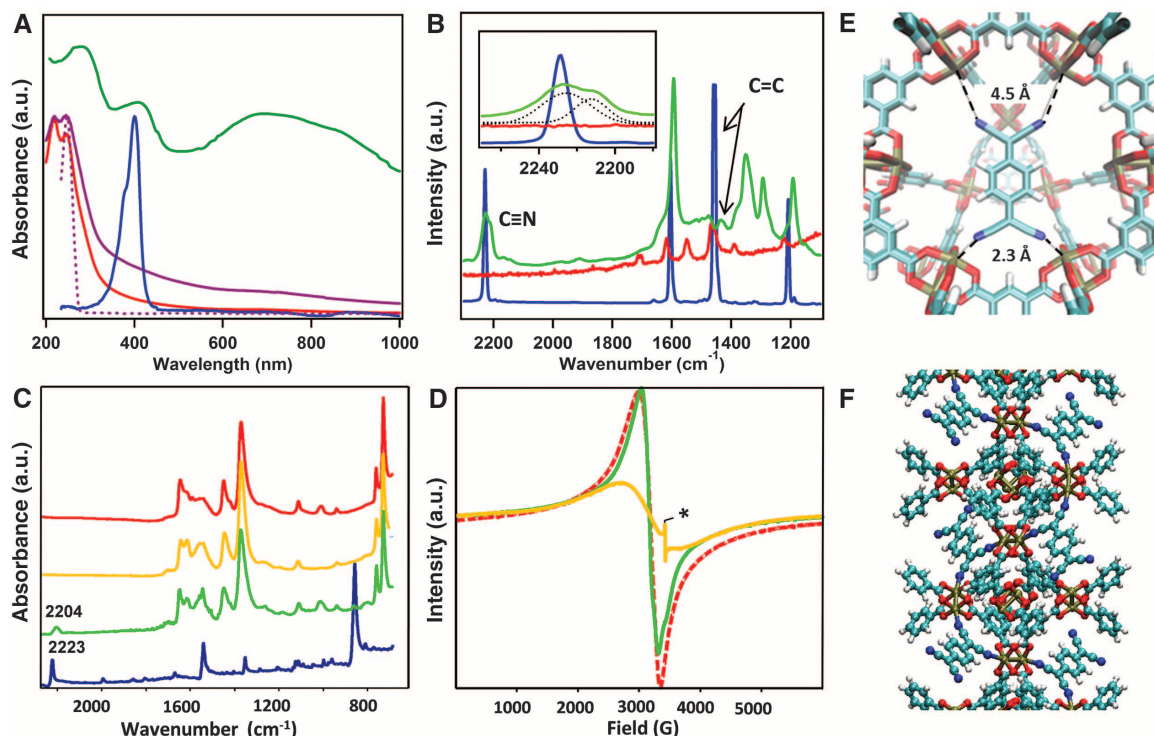


Fig. 3. Evidence for interaction between TCNQ and the MOF. (A) Transmission UV-vis spectra collected for a $\text{Cu}_3(\text{BTC})_2 \cdot x\text{H}_2\text{O}$ film on borosilicate substrate before (red) and after adsorption with TCNQ (green) or H4-TCNQ (purple), and for TCNQ crystals deposited onto a glass slide (blue). (B) Raman spectra collected for a $\text{Cu}_3(\text{BTC})_2 \cdot x\text{H}_2\text{O}$ film on borosilicate substrate before (red) and after adsorption of TCNQ (green), and for TCNQ crystals deposited onto a glass slide (blue). (C) Infrared spectra collected for $\text{Cu}_3(\text{BTC})_2 \cdot x\text{H}_2\text{O}$ (red), $\text{Cu}_3(\text{BTC})_2$ (yellow), TCNQ@ $\text{Cu}_3(\text{BTC})_2$ (green), and TCNQ powder (blue). (D) Room-temperature continuous-wave EPR spectra of activated $\text{Cu}_3(\text{BTC})_2$ (yellow), $\text{Cu}_3(\text{BTC})_2$ stirred in methanol (red dashed), and $\text{Cu}_3(\text{BTC})_2$ stirred in methanol containing TCNQ (green). The asterisk denotes an unidentified organic radical signal observed only in the activated $\text{Cu}_3(\text{BTC})_2$ sample. (E) Minimum-energy configuration for TCNQ@ $\text{Cu}_3(\text{BTC})_2$



obtained from ab initio calculations. (F) Possible configuration that would provide a conductive channel through the MOF unit cell. Atom color code for (E) and (F) as in Fig. 1.

the framework and TCNQ, in reasonable agreement with the value inferred from the Raman spectra. The observation of partial charge transfer is further supported by room-temperature EPR spectra obtained from TCNQ@ $\text{Cu}_3(\text{BTC})_2$ (Fig. 3D), which display no evidence of TCNQ radical anions (22). Partial charge transfer is characteristic of many conducting TCNQ salts; however, in contrast to the well-studied CuTCNQ , in which $\text{Cu}(+1)$ predominates, Cu in $\text{Cu}_3(\text{BTC})_2$ remains in a +2 state after infiltration, as revealed by XPS (fig. S4).

The importance of guest-host interactions was further probed by replacing TCNQ with its fully hydrogenated counterpart, H4-TCNQ [cyclohexane-1,4-diylidene)dimalononitrile], which lacks a conjugated π electron network, and F4-TCNQ (2,3,5,6-tetrafluoro-7,7,8,8-tetracyanoquinodimethane), which has a similar HOMO-LUMO (highest occupied molecular orbital–lowest unoccupied molecular orbital) gap but higher electron affinity relative to TCNQ. Elemental analysis indicates that the loading of H4-TCNQ is similar to that of TCNQ (i.e., about 1 H4-TCNQ molecule per pore). The I - V curve (Fig. 2A) for H4-TCNQ@ $\text{Cu}_3(\text{BTC})_2$ is essentially the same as that of the uninfiltrated, nonconducting MOF. The UV-vis spectrum also lacks the characteristic bands indicative of charge transfer in TCNQ@ $\text{Cu}_3(\text{BTC})_2$ (Fig. 3A). These results illustrate that the availability of guest molecule orbitals that can accept charge, as is the case in TCNQ but not H4-TCNQ, is important for achieving

high conductivity. F4-TCNQ@ $\text{Cu}_3(\text{BTC})_2$ is not as conductive as TCNQ@ $\text{Cu}_3(\text{BTC})_2$. We view this result as semiquantitative, however, because F4-TCNQ is volatile, and unlike TCNQ@ $\text{Cu}_3(\text{BTC})_2$, the conductivity was not stable with time. Nonetheless, the lower conductivity measured immediately after infiltration suggests that the high electron affinity of this molecule inhibits electron mobility.

Finally, ab initio calculations suggest a possible mechanism for the appearance of conductance in TCNQ@ $\text{Cu}_3(\text{BTC})_2$ hybrids. As illustrated in Fig. 3E, the calculations predict that TCNQ binds strongly to the MOF (binding energy of 83.9 kJ/mol) and that four such molecules create a continuous path through the unit cell (Fig. 3F and movie S1). Our calculations for molecular clusters comprising two copper dimer groups (MOF secondary building units) bridged by a TCNQ molecule show that the bridging TCNQ inserts unoccupied molecular orbitals into the MOF HOMO-LUMO gap (fig. S5), producing the new charge transfer band in the visible spectrum and enabling electronic coupling between the MOF and TCNQ. Moreover, computed values of H_{AB} , the electronic coupling matrix element for electron transfer from the MOF cluster to TCNQ [i.e., $\text{TCNQ@Cu}_3(\text{BTC})_2 \rightarrow \text{TCNQ@Cu}_3(\text{BTC})_2$], combined with the value of the activation energy obtained from the temperature dependence of the conductivity, allow us to evaluate the extent of donor-acceptor coupling using the quantity $2H_{AB}/\lambda$ (where λ is the re-

organization parameter) defined by Brunschwig *et al.* (19). We find $2H_{AB}/\lambda = 1.21$ for TCNQ@ $\text{Cu}_3(\text{BTC})_2$, identifying this material as a class III system according to the Robin-Day classification scheme (19). These calculations also predict that electronic coupling in F4-TCNQ@ $\text{Cu}_3(\text{BTC})_2$ is intermediate between H4-TCNQ and TCNQ itself. The order of H_{AB} values is H4-TCNQ < F4-TCNQ < TCNQ (0.19 eV < 1.03 eV < 2.32 eV)—a trend that is consistent with the observed conductivities.

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Supplementary Materials

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Two Y Genes Can Replace the Entire Y Chromosome for Assisted Reproduction in the Mouse

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The Y chromosome is thought to be important for male reproduction. We have previously shown that, with the use of assisted reproduction, live offspring can be obtained from mice lacking the entire Y chromosome long arm. Here, we demonstrate that live mouse progeny can also be generated by using germ cells from males with the Y chromosome contribution limited to only two genes, the testis determinant factor *Sry* and the spermatogonial proliferation factor *Eif2s3y*. *Sry* is believed to function primarily in sex determination during fetal life. *Eif2s3y* may be the only Y chromosome gene required to drive mouse spermatogenesis, allowing formation of haploid germ cells that are functional in assisted reproduction. Our findings are relevant, but not directly translatable, to human male infertility cases.

The mammalian Y chromosome, once thought to be a genetic wasteland (1), is now known to encode a battery of genes, many of which are thought to be involved in male reproduction (2). A substantial amount of work has been done to define which genes are important for maintaining sperm function under normal, in vivo, conditions. In the era of assisted reproduction technologies (ART), it is now possible to bypass several steps of normal human fertilization using immotile, nonviable, or even immature sperm. We have shown that infertile male mice lacking the entire Y chromosome long arm can generate live offspring when their severely morphologically abnormal sperm are delivered into oocytes via intracytoplasmic sperm injection (ICSI) (3). In these mice, the Y chromosome is reduced from 78 Mb to ~2 Mb and encodes only seven genes and three gene families (XY*^XSr^a in fig. S1).

In most mammals, including humans and mice, testis determination is regulated by *Sry*, which directs developing gonads to male differ-

entiation (4–6). Upon transgenic addition of *Sry*, mice with one X chromosome (XO^{Sry}) develop testes that are populated with spermatogonia, male germ cells that have the potential to undergo differentiation and initiate spermatogenesis. In the absence of other Y chromosome genes, these spermatogonia undergo proliferation arrest, and the meiotic and postmeiotic stages of spermatogenesis are absent (7). Transgenic addition of individual missing Y genes led to the identification of *Eif2s3y* as the gene that restored normal spermatogonial proliferation (7). In XO^{Sry} males transgenic for *Eif2s3y*, spermatogenesis was shown to complete meiotic prophase and the first meiotic division before the cells arrested as secondary spermatocytes, with the occasional production of spermatid-like cells (7, 8). Here, we tested whether these spermatid-like cells were functional in assisted reproduction and what other components of the Y chromosome help to increase development of functional gametes.

We first examined mice with the Y gene complement limited to two transgenically derived genes, autosomally located *Sry* and X chromosome located *Eif2s3y* (X^EO^{Sry} in fig. S1) (9). These mice had testes smaller than wild-type XY males (fig. S2) but populated with germ cells. Analysis of testicular sections confirmed that sper-

matogenesis was ongoing, which allowed development of germ cells with spermatid-like morphology (Fig. 1A, arrowheads), similar to those observed earlier (7, 10). The occurrence of these spermatids was low, and their development was restricted to steps 5 to 7 of spermatid development, with the occasional presence of step 8 to 9 spermatids [Fig. 1A (inset) and fig. S3]. We also observed secondary changes to seminiferous tubules, with increased incidence of dying cells, multinucleate bodies, and vacuoles (Fig. 1B). These changes increased progressively as the males aged and ultimately led to Sertoli-cell-only (SCO) syndrome tubules (Fig. 1C).

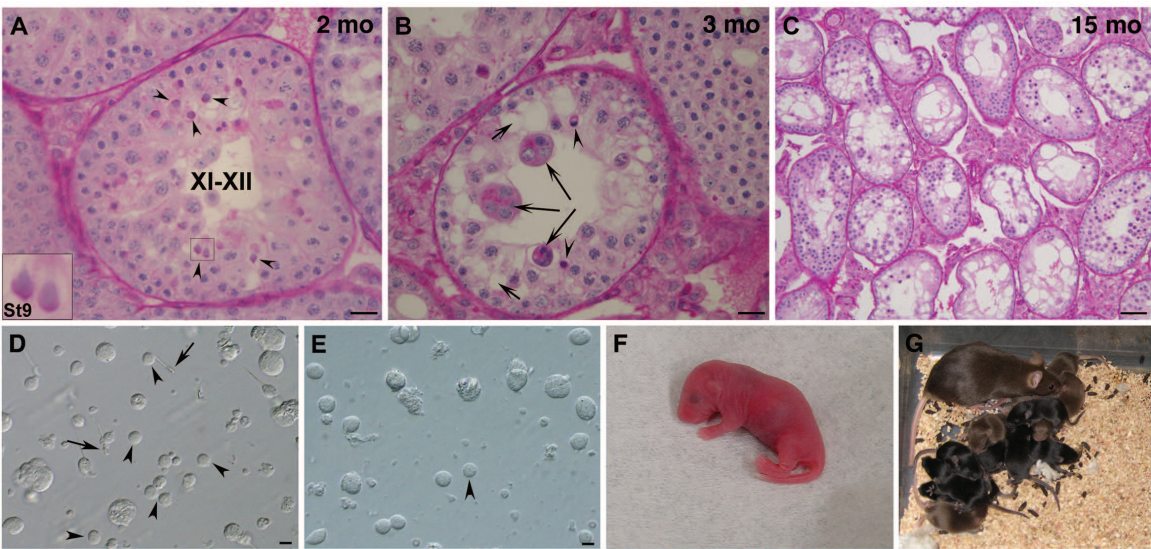
We next tested the function of the spermatid-like cells from X^EO^{Sry} in assisted reproduction. Round, spermatid-like cells could be found in testicular cell suspensions from all males used in ART trials, although these cells were rare and their morphology was often slightly abnormal (increased size, less pronounced nuclei, and rough rather than smooth surface) when compared with spermatids from control XY males (Fig. 1, D and E). Nevertheless, when we performed round spermatid injection (ROSI), the oocytes were successfully fertilized, as evidenced by the development of two pronuclei and extrusion of the second polar body, as well as subsequent cleavage (fig. S4). When the developed two-cell embryos were transferred into the oviducts of recipient females, live offspring were obtained (Table 1 and Fig. 1F). Three out of four males that provided spermatids for injections successfully sired offspring. The efficiency of ROSI with X^EO^{Sry} males was significantly lower than with XY controls (9% versus 26%) (Table 1). All the progeny had the genotypes as expected when derived from X^EO^{Sry} fathers and were healthy; those bred were fertile (Fig. 1G, fig. S5, and supplementary text).

An unpaired sex chromosome leads to meiotic arrest and apoptosis (11), so partial meiotic failure in X^EO^{Sry} males was not unexpected. The few spermatids that could be found in the testes could be the cells that “leaked” through the meiotic arrest, i.e., finished meiosis and were haploid. Alternatively, these could be the cells that developed spermatid-like morphology without undergoing the second meiotic division (8, 10).

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Fig. 1. Testis histology, round spermatids, and live offspring. (A) Males with only two Y genes, *Eif2s3y* and *Sry*, have meiotic and postmeiotic arrests that only occasionally allow formation of spermatids (arrowheads) that are frequently delayed and do not develop beyond St8/9. (B) Tubule degeneration with formation of “multinucleate bodies” (long arrows), vacuoles (short arrows), and dying and/or apoptotic germ cells (arrowheads) is frequently observed. (C) SCO syndrome-like phenotype in an old male. (D) Testicular suspension from wild-type males contains testicular sperm (arrows) and many round spermatids (arrowheads) with clear morphological features. (E) Males with two Y genes have substantially fewer germ cells in testicular cell suspension, with no sperm and very few round spermatids (arrowhead). These spermatids are functional in ROSI. (F) ROSI pup obtained after transfer of embryos generated with spermatids



from a male with only two Y genes. (G) An adult female developed from the pup shown in (F) with her own litter. Roman and arabic numerals in (A) are tubule stages and steps of spermatid development (St), respectively (see fig. S3). Scale bars, 16 μ m (A and B), 50 μ m (C), and 10 μ m (D and E); inset, $\times 3$ magnification; mo, months of age.

Table 1. The results of round spermatid injection (ROSI) with spermatids from males with limited Y gene complement. For Y gene contribution, see fig. S1. Percentages of live offspring and implants were calculated from embryos transferred. Male ages ranged from 63 to 229 days. Statistical significance (Fisher’s exact test): ^a*P* < 0.01 and ^b*P* < 0.001 versus XY^{RIII} control. *Sxr^b*: *Sry*, *Zfy2/1*, *H2al2y*, *Rbmy* cluster (reduced).

Male genotype	Y gene contribution	Live offspring % (no.)	Implants % (no.)
X ^E OSry	<i>Eif2s3y</i> and <i>Sry</i>	9.1 (12/132) ^a	29.5 (39/132) ^b
X ^E Y* ^X Sry	<i>Eif2s3y</i> and <i>Sry</i>	5.7 (13/227) ^b	27.3 (62/227) ^b
X ^E Sxr ^b O	<i>Eif2s3y</i> and <i>Sxr^b</i>	20.0 (24/120)	45.8 (55/120) ^a
X ^E Sxr ^b Y* ^X	<i>Eif2s3y</i> and <i>Sxr^b</i>	16.0 (24/150)	37.3 (56/150) ^b
XY ^{RIII} control	Intact Y	26.0 (19/73)	69.9 (51/73)

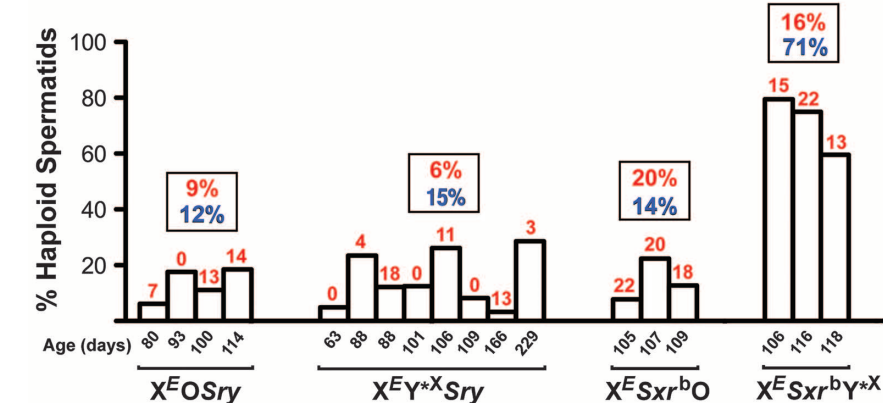


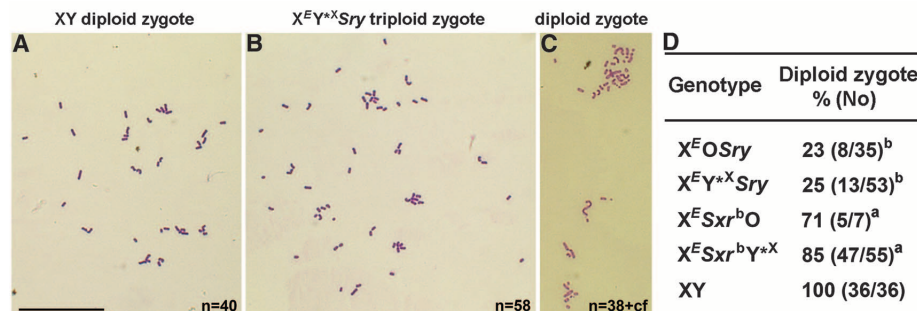
Fig. 2. Incidence of haploid spermatids. Each graph bar represents an individual male providing testicular cells for analysis; the numerals above show the percentage of pups obtained after ROSI. The data in rectangular boxes represent average percentage of haploid spermatids (blue) and ROSI offspring (red) for each genotype. Control male with intact Y chromosome had 97% (67 out of 69) of spermatids haploid. X^ESxr^bY*^X males had higher average incidence of haploid spermatids than other genotypes (*P* < 0.01).

Spermatid nuclear DNA content (Fig. 2 and fig. S6) and zygotic chromosome analyses (Fig. 3) revealed that the great majority of spermatids from X^EOSry males were diploid and yielded triploid zygotes, which would explain the poor ROSI success. In order to overcome the problem of meiotic block arising from X chromosome univalence, we used males in which a minute Y*^X chromosome (fig. S1) was added to provide a second pairing region (PAR) for PAR-PAR chromosome synapsis (12). In these X^EY*^XSry males (fig. S1), successful pairing of Y*^X and X was observed in 85% of pachytene spermatocytes (fig. S8). However, the testicular phenotype did not improve (figs. S2 and S7, A to C). The proportion of live offspring obtained after injection was similarly low as with X^EOSry males (Table 1), and only five out of eight males that provided cells for ROSI sired offspring. We therefore tested for ploidy and demonstrated that most of the X^EY*^XSry spermatids were diploid (Fig. 2 and fig. S6) and that most zygotes after ROSI were triploid (Fig. 3). Thus, overcoming X chromosome univalence in X^EY*^XSry males did not allow overcoming meiotic arrest and increasing ROSI success (see supplementary text).

We next asked whether other Y chromosome genes may be beneficial for spermatid function in assisted reproduction. To address this, we used males in which the *Sry* transgene driving sex determination was replaced with the sex reversal factor *Sxr^b* (X^ESxr^bO and X^ESxr^bY*^X in fig. S1). In these males, the X chromosome carries an *Eif2s3y* transgene necessary for spermatogonial proliferation (7), together with the *Sxr^b* encoding for *Zfy2/1*, *Sry*, *H2al2y*, and the *Rbmy* gene cluster.

Fig. 3. Zygote chromosome analysis after ROSI.

(A) Normal diploid mouse zygote generated after ROSI with XY male contains 40 chromosomes. (B) Triploid zygote obtained after ROSI with spermatids from a male with two Y genes (~60 chromosomes). (C) Diploid zygote after ROSI with spermatids from male of the same genotype (~40 chromosomes). (D) Incidences of diploid zygotes generated after ROSI. cf, chromosome fragments. n = chromosome number. ^a $P < 0.05$, ^b $P < 0.001$ versus XY control. Scale bar, 50 μ m.



The testes from $X^E Sxr^b O$ and $X^E Sxr^b Y^* X$ males were larger than from $X^E O Sry$ and $X^E Y^* X Sry$ but smaller than in wild-type XY males (fig. S2). The incidence of round spermatids increased only in $X^E Sxr^b Y^* X$ (fig. S3). In both male types, spermatid development was more advanced, with clear elongation up to step 10, occasional presence of step 11 to 12 spermatids, and even sporadic development to mature testicular sperm (fig. S7, D and G). There were secondary changes to the seminiferous epithelium (fig. S7, E, F, H, and I), albeit in $X^E Sxr^b Y^* X$ males less pronounced than in the other genotypes. Zygote chromosome analysis showed that most of the zygotes after ROSI were diploid (71 to 85%) (Fig. 3), and the ROSI outcome was significantly improved, with all tested males (three per genotype) yielding live offspring with the rate now reaching 20 and 16% (Table 1). Nevertheless, the frequency of haploid spermatids in $X^E Sxr^b O$ males remained low (Fig. 2 and fig. S6). The discrepancy between the ploidy of the spermatids and zygotes raised the possibility that the second meiotic division took place in the oocytes. This suggests that Sxr^b encodes a gene or genes that promote the second meiotic division when all chromosomes are paired and enables overcoming of meiotic arrest of the diploid spermatid in the oocyte when an X chromosome pairing partner is missing (see supplementary text).

We have shown that only two mouse Y chromosome genes, *Sry* and *Eif2s3y*, are necessary for the development of male haploid germ cells that are sufficient for successful reproduction and yield live offspring. This minimal Y gene complement must be enough to ensure correct male-specific methylation and proper formation of any other epigenetic modifications in the paternal genome that are required for embryogenesis.

That *Sry* is one of the two genes is not surprising, as it drives testis determination (4–6). Mouse *Sry* expression and translation occur very briefly during fetal development. In adult testes, *Sry* transcripts are thought to be aberrant and not translatable (13, 14), although their role as epigenetic regulator(s) cannot be excluded (15). Nevertheless, it is reasonable to conclude that *Sry* plays a role primarily during sex determination.

This suggests that the second Y gene, *Eif2s3y*, is the only gene necessary to drive spermatogenesis through the first meiotic division and with the occasional meiotic progression to form haploid

round spermatids. *Eif2s3y* is a Y-encoded subunit 3 of the eukaryotic translation initiation factor 2. It is ubiquitously expressed and, in the testis, plays a role in spermatogonial proliferation (7). The *Eif2s3y* gene has been conserved on the Y chromosome during eutherian evolution, but there is no Y-linked copy of *Eif2s3* detected in any of the simian primates, including humans (16).

In men, spermatogonial proliferation arrest results most often from deletions of *AZF*a (azoospermia; SCO syndrome), and it has been related to a loss of *DDX3Y* (17–19). In both men and mice, the *Eif2s3* and *Ddx3* genes belong to the family of widely expressed genes encoding proteins involved in initiation of mRNA translation at the ribosome. These genes have X homologs that escape X-inactivation, and the Y and X copies are suspected to be, at least in part, interchangeable, with the Y copy conserved to provide two doses of gene product in both male and female. The loss of the mouse Y-encoded *Ddx3y* is not detrimental for spermatogenesis because of the compensation provided by an X copy retrotransposed on an autosome (20). Analogically, in men, the presence of a retrotransposed X copy of *EIF2S3X*, in addition to *EIF2S3X* itself, explains why the loss of the Y copy was still permissive for spermatogenesis. Although there is no human copy of *Eif2s3y*, men have a Y-encoded copy of the translation factor EIF1A (eukaryotic translation initiation factor 1A, Y-linked) (21), which likely acts as part of a multiprotein complex that includes EIF2S3X, as well as other EIF family members. Note that EIF1AY is found in the *AZF*b region, and its diminished expression sporadically contributes to azoospermia (22).

At present, our findings in mice do not translate directly to humans. ROSI is still considered experimental in human ART because of concerns regarding the safety of injecting immature germ cells and technical difficulties (23). In spite of this, some children have already been born (24, 25), and those were healthy. As we learn more about the effects and improve technical aspects of ROSI, this method may become more acceptable. Indeed, studies on ROSI effects in mice have been encouraging (26). Thus, our study may bear importance for clinicians working in ART clinics by supporting the possibility that ROSI may be a viable option for overcoming infertility in men with nonobstructive azoospermia.

Considering that we have obtained live offspring using germ cells from males with only two Y chromosome genes, one could question the importance of the Y chromosome in male reproduction. We believe that the answer lies in defining the need. Human Y chromosome is not on the way to oblivion, as has been implied in the past (27), and its genetic information is undoubtedly important for many aspects of reproduction involving the development of mature sperm and its function in normal fertilization (28). Most of the mouse Y chromosome genes are involved in spermatogenesis and sperm function and, as such, are necessary for normal fertilization (29, 30). However, when it comes to assisted reproduction, our mouse study proves that the Y chromosome contribution can be brought to a bare minimum consisting of *Sry* and *Eif2s3y*. Indeed, it may well be possible to eliminate mouse Y chromosome altogether if appropriate replacements are made for those two genes.

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Supplementary Materials

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Table S1

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Targeted Therapy Resistance Mediated by Dynamic Regulation of Extrachromosomal Mutant EGFR DNA

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Intratumor heterogeneity contributes to cancer drug resistance, but the underlying mechanisms are not understood. Single-cell analyses of patient-derived models and clinical samples from glioblastoma patients treated with epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) demonstrate that tumor cells reversibly up-regulate or suppress mutant EGFR expression, conferring distinct cellular phenotypes to reach an optimal equilibrium for growth. Resistance to EGFR TKIs is shown to occur by elimination of mutant *EGFR* from extrachromosomal DNA. After drug withdrawal, reemergence of clonal *EGFR* mutations on extrachromosomal DNA follows. These results indicate a highly specific, dynamic, and adaptive route by which cancers can evade therapies that target oncogenes maintained on extrachromosomal DNA.

The majority of targeted therapies have not produced substantial survival benefits for most cancer patients (1, 2). A variety of re-

sistance mechanisms have been described, including incomplete target suppression, second-site mutations, and activation of alternative kinases to maintain signal flux to downstream effector pathways (1–3). Thus, most efforts are now aimed at developing better drugs or better drug combinations to more fully suppress the target oncogenes and their downstream signals. Changes in the cellular composition of tumors, particularly in response to targeted treatment, could facilitate such a resistance mechanism and thereby dictate patient response.

In glioblastoma (GBM), the most common malignant primary brain cancer of adults, the epidermal growth factor receptor (*EGFR*) is frequently mutated, commonly giving rise to the constitutively active oncogenic variant *EGFRvIII* (4, 5). *EGFRvIII* potently accelerates tumor growth by cell-autonomous and intercellular signaling mechanisms (6), but it also makes tumor cells that express it more sensitive to EGFR tyrosine kinase inhibitors (TKIs) (7, 8). In clinical GBM samples, the level of *EGFRvIII* protein expression varies widely among cells within the tumor mass (6, 9–15). The potential con-

tribution of heterogeneous *EGFRvIII* expression to EGFR TKI resistance in GBM (16) is not understood.

To determine whether *EGFRvIII* heterogeneity contributes to EGFR TKI resistance, single-cell analyses of a patient-derived *EGFRvIII*-expressing xenograft model (GBM39) (17) were performed. GBM39 cells stably express firefly luciferase (ff-LUC), enabling definitive tumor cell identification (fig. S1A). Quantitative microfluidic image cytometry (MIC) (18) demonstrated detectable levels of *EGFRvIII* protein in 60% (±5%) of tumor cells (fig. S1B). The *EGFRvIII*-expressing tumor cells (*EGFRvIII*^{High}) demonstrated increased phosphatidylinositol 3-kinase–Akt–mammalian target of rapamycin (PI3K–Akt–mTOR) signaling (Fig. 1A and fig. S2), elevation in tumor cell proliferation by a factor of 4 (Fig. 1B and fig. S2), a lower basal apoptotic rate by a factor of 15 (Fig. 1C and fig. S2), and increased glucose uptake (Fig. 1D) relative to the GBM cells lacking detectable *EGFRvIII* protein (*EGFRvIII*^{Low}) (Fig. 1, D and E). Further, the *EGFRvIII*^{High} tumor cells showed enhanced cell death in response to the EGFR TKI erlotinib (Fig. 1F).

To determine the effect of an EGFR TKI on *EGFRvIII* population dynamics, mice bearing tumors were treated daily with oral erlotinib (150 mg per kg of weight). Erlotinib treatment initially caused 80% tumor shrinkage (response) (blue line in Fig. 1G), shifting the composition of tumors from being predominantly *EGFRvIII*^{High} to predominantly *EGFRvIII*^{Low} tumor cells (Fig. 1H and fig. S3). This shift in the *EGFRvIII* population dynamics was maintained, even after tumors developed resistance to continued erlotinib treatment (resistant) [Fig. 1G (red line) and H, and fig. S3], and was also detected in another patient-derived ex vivo neurosphere culture, HK296 (fig. S4). Most important, in tumor tissue from GBM patients treated for 7 to 10 days with the EGFR/HER2 inhibitor lapatinib, the relative fraction of *EGFRvIII*^{High} tumor cells dramatically declined relative to each patient's pretreatment sample (Fig. 1, I and J). Of note, this analysis was confined to patients whose posttreatment tumor tissue showed reduced EGFR phosphorylation relative to the pretreatment sample. We did not detect any decrease in *EGFRvIII* level in the two available GBMs in which no decrease in phospho-EGFR was seen after lapatinib treatment.

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Unexpectedly, EGFRvIII^{High}/EGFRvIII^{Low} GBM subpopulations sorted by fluorescence-activated cell sorting (FACS) proved to be equally tumorigenic, giving rise to heterogeneous tumors (Fig. 2, A and B). Seeds containing ~200, 2000, or 20,000 cells of FACS-sorted EGFRvIII^{High}/EGFRvIII^{Low} subpopulations generated subcutaneous tumors of similar size, containing the same ratio of EGFRvIII^{High}/EGFRvIII^{Low} cells as the original tumor (Fig. 2C). FACS-sorted pure populations of EGFRvIII^{High} and EGFRvIII^{Low} GBM cells plated at 2 to 5 cells per well, or even as single cells per well, gave rise to colonies containing a similar ratio of EGFRvIII^{High}/EGFRvIII^{Low} cells (Fig. 2, D to F). These findings are consistent with a stochastic state transition model, in which distinct tumor subpopulations regenerate the phenotypic equilibrium characteristic of the original tumor (19). Erlotinib treatment completely suppressed this state transition and maintained the

population in an EGFRvIII^{Low} state, and it continued to do so long after any tumor cell death was observed (fig. S5).

To determine the mechanism by which GBM cells modulate EGFRvIII protein levels during erlotinib resistance, we generated a reversible EGFR TKI resistance model by continuous treatment of GBM cells with erlotinib in GBM39 cells in neurosphere culture, followed by drug withdrawal (Fig. 3, A to C), and examined the level, sequence, and subnuclear localization of EGFRvIII DNA. EGFRvIII arises from an in-frame genomic deletion of exons 2 to 7 of the EGFR gene and has been thought to reside primarily on small circular extrachromosomal fragments of DNA called double-minute (DM) chromosomes (20–22). Fluorescent in situ hybridization (FISH) of naïve ($n = 15$ metaphases), erlotinib-resistant ($n = 15$ metaphases), and drug-removed ($n = 10$ metaphases) GBM39 metaphase cells with EGFR and centromere 7–

specific DNA probes revealed abundant EGFR⁺ extrachromosomal DNA in naïve and drug-removed tumor cells and a complete loss of these EGFR⁺ extrachromosomal DNA elements in erlotinib-resistant GBM cells (Fig. 3D). No changes in chromosomal EGFR copy number were detected between naïve, erlotinib-resistant, or drug-removed GBM cells. The overall FISH signal patterns were confirmed by analysis of more than 100 interphase nuclei from each of the three conditions (fig. S7). The loss of EGFR⁺ extrachromosomal DNA in erlotinib resistance was specific, because these cells still contained abundant extrachromosomal DNA elements (fig. S6), including MDM2⁺ DMs, which were identified by FISH and polymerase chain reaction (PCR)–Southern blot analysis. The MDM2⁺ DM copy number rose with erlotinib treatment and remained elevated, even after drug withdrawal (fig. S8). In each erlotinib-resistant metaphase or interphase cell, a single

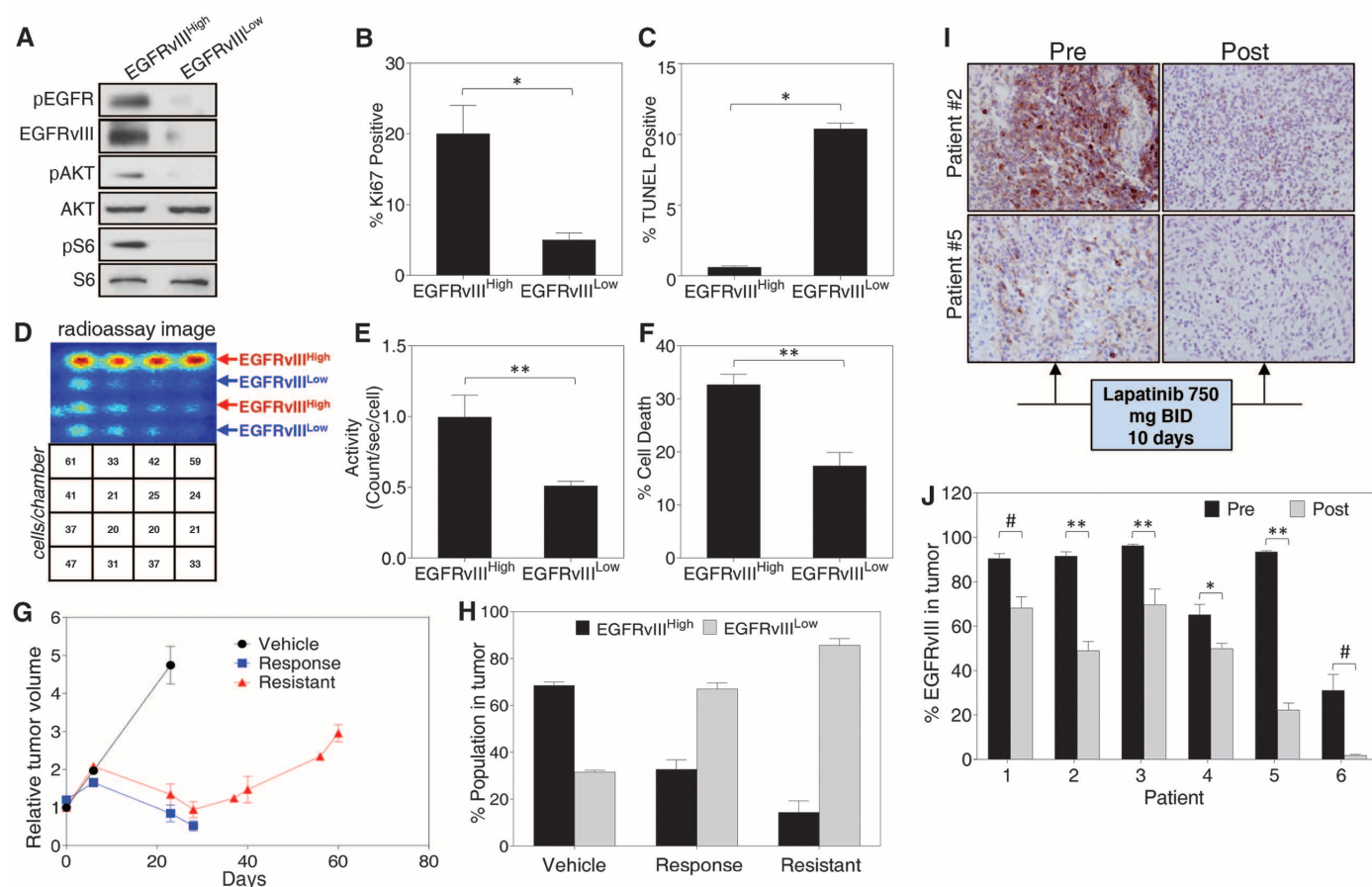


Fig. 1. Resistance to EGFR TKIs in preclinical models and GBM patients treated with an EGFR TKI is associated with a decreasing ratio of EGFRvIII^{High}/EGFRvIII^{Low} tumor cells. (A) FACS-sorted EGFRvIII^{High} and EGFRvIII^{Low} cells obtained from GBM39 differ in their PI3K-Akt-mTOR activity as determined by immunoblotting. (B) Immunofluorescence (IF) for EGFRvIII and Ki-67 on isolated GBM39 tumor cells shows differences in basal proliferative rate between EGFRvIII^{High} and EGFRvIII^{Low} tumor cells. * $P < 0.005$. (C) Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) stain and EGFRvIII IF indicate a higher basal apoptosis in EGFRvIII^{Low} tumor cells. * $P < 0.005$. (D and E) Radiopharmaceutical imaging chip analysis of

¹⁸F-fluorodeoxyglucose from FACS-sorted EGFRvIII^{High} and EGFRvIII^{Low} cells indicates higher glucose uptake in EGFRvIII^{High} cells. ** $P < 0.05$. (F) FACS-sorted EGFRvIII^{High} and EGFRvIII^{Low} were treated with erlotinib (5 μ M) for 24 hours, and cell viability was determined by trypan blue exclusion assay. ** $P < 0.05$. (G and H) Resistance to erlotinib in GBM39 xenografts ($n = 4$ mice per group). During initial response (blue curve) and at the time of resistance (red curve), there is a relative loss of EGFRvIII-expressing tumor cells. (I and J) In GBM patients, 10 days of treatment with the EGFR tyrosine kinase inhibitor lapatinib reduces EGFRvIII expression relative to pretreatment levels. * $P < 0.01$; ** $P < 0.0001$; # $P < 0.001$. All values are mean $\pm$ SEM. P values were obtained from unpaired t test.

marker chromosome made up of homogeneous staining regions (HSRs) positive for *EGFR*, but lacking the chromosome 7-specific centromere sequences, was detected. Similar HSR-like *EGFR*⁺ marker chromosomes were detected in naïve and drug-removed GBM metaphases, raising the possibility that these bodies may serve as a latent reservoir for EGFRvIII in erlotinib-resistant GBM cells (Fig. 3D and fig. S9).

To confirm that the *EGFR*⁺ extrachromosomal elements were *EGFRvIII*, we performed Southern blot, PCR, and sequencing analyses of low-molecular-weight extrachromosomal DNA (Fig. 3, E to I). A 4.1-kb band in BamHI-digested low-molecular-weight DNA indicative of *EGFRvIII* was detected in naïve and drug-removed GBM39 cells but not erlotinib-resistant cells (Fig. 3, E and F). The intronic breakpoints that give rise to the *EGFRvIII* deletion are not consistent, varying between individuals (23). Therefore, we designed primers mapping at each of the 17 BamHI restriction sites spanning the region of interest. PCR and Southern blot analyses identified a genomic deletion confirmed to be *EGFRvIII* by sequencing of cloned fragments (fig. S10 and Fig. 3, E to I). We identified the intronic breakpoints giving rise to *EGFRvIII* in two additional patient-derived GBM ex vivo neurosphere cultures (GBM6 and HK296) (fig. S10) (24, 25) and measured extrachromosomal *EGFRvIII* DNA copy number

in naïve and erlotinib-resistant cells, including after drug withdrawal, by a quantitative PCR assay. Consistent with the effect of erlotinib in GBM39 cells, continuous EGFR TKI treatment caused almost complete loss of extrachromosomal *EGFRvIII* DNA and erlotinib resistance (Fig. 4A and fig. S11). Remarkably, cessation of erlotinib treatment for as little as 72 hours in GBM6 and HK296 markedly increased extrachromosomal *EGFRvIII* DNA and resensitized tumor cells to erlotinib-induced cell death (fig. S11).

The availability of two pairs of matched formalin-fixed, paraffin-embedded tissue sections from patients pre- and post-lapatinib treatment (patients 2 and 3 from Fig. 1, I and J) enabled us to perform FISH using probes for EGFR (which recognizes EGFRvIII) and centromere 7. In both patients, a nearly 80% reduction in EGFR FISH-positive signals after lapatinib treatment was detected (Fig. 4B), thus indicating the clinical relevance of loss of extrachromosomal EGFRvIII DNA as an EGFR TKI resistance mechanism. In this cohort of patients, intratumoral lapatinib levels sufficient to cause tumor cell death (2000 to 3000 nM) were not achieved in the tumor samples (7). However, 1500 nM of lapatinib, an intratumoral level observed in at least some of these patients, was sufficient to cause highly significant reduction of extrachromosomal EGFRvIII DNA in GBM39, GBM6, and HK296 neurospheres in

culture (fig. S12), suggesting that the significant reduction seen in patients 2 and 3 was a consequence of lapatinib treatment.

Analysis of an additional four matched sets of EGFRvIII-positive tumor tissue from GBM patients before and after treatment with conventional therapy (temozolomide and radiation) showed no detectable difference in extrachromosomal EGFR FISH-positive DNA levels (Fig. 4C). Taken together, these results indicate that loss of extrachromosomal *EGFRvIII* DNA is a general and clinically relevant EGFR TKI resistance mechanism in GBM.

It is unusual for EGFRvIII to be homogeneously expressed in a tumor, despite the selective growth advantage conferred to individual GBM cells (Fig. 1, B, C, E, and F). EGFRvIII possibly imposes a cost to tumor cells, potentially by increasing nutrient requirements (Fig. 1D). Notably, the EGFR TKI resistance mechanism identified here is entirely distinct from the mechanism by which GBMs maintain EGFRvIII heterogeneity in the absence of treatment. Extrachromosomal *EGFRvIII* DNA copy number remains elevated in the treatment-naïve EGFRvIII^{Low} cells (fig. S13), consistent with an epigenetic regulatory mechanism of EGFRvIII heterogeneity (15). Taken together, these results highlight the exquisite specificity of reversible loss of extrachromosomal *EGFRvIII* DNA as a GBM EGFR TKI resistance mechanism.

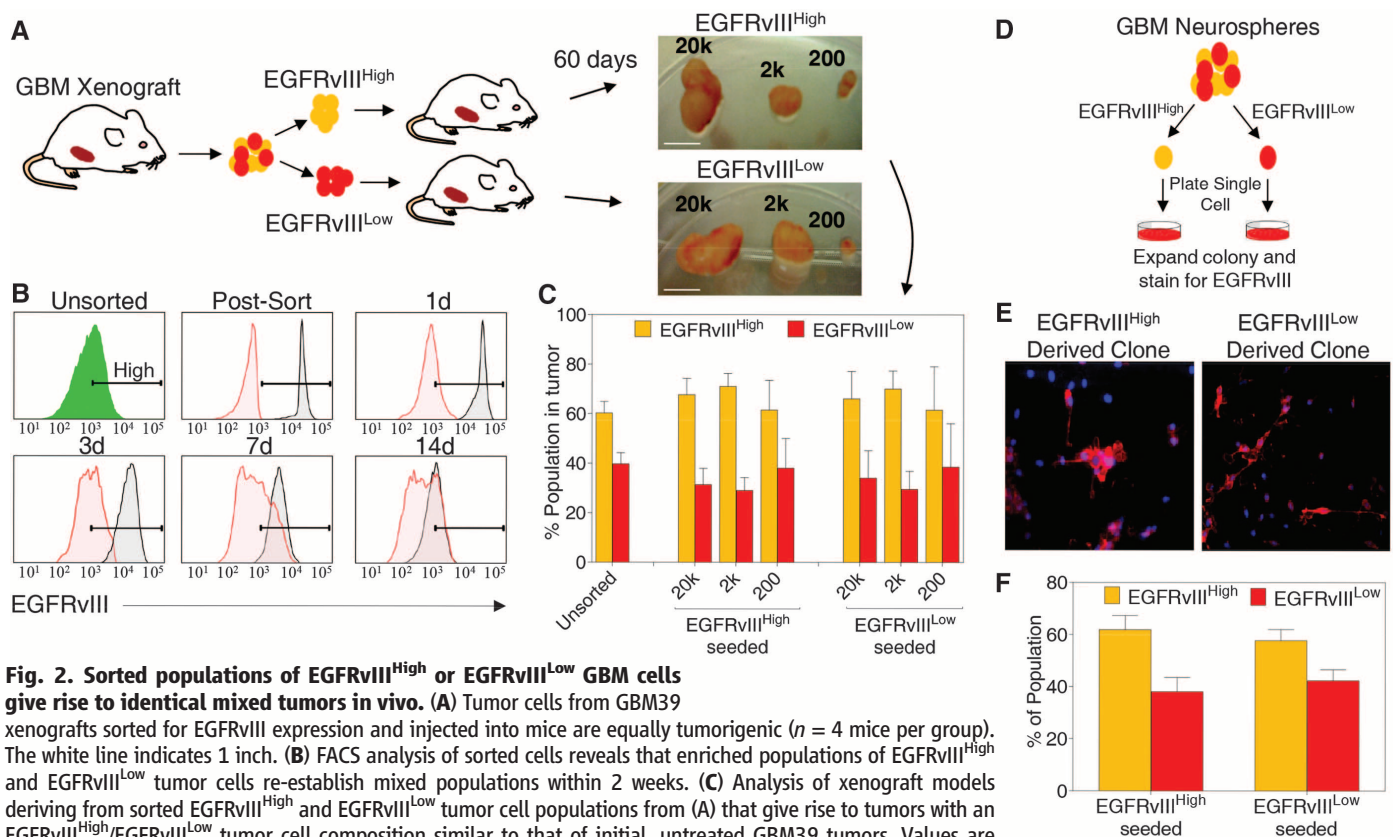


Fig. 2. Sorted populations of EGFRvIII^{High} or EGFRvIII^{Low} GBM cells give rise to identical mixed tumors in vivo. (A) Tumor cells from GBM39 xenografts sorted for EGFRvIII expression and injected into mice are equally tumorigenic ($n = 4$ mice per group). The white line indicates 1 inch. (B) FACS analysis of sorted cells reveals that enriched populations of EGFRvIII^{High} and EGFRvIII^{Low} tumor cells re-establish mixed populations within 2 weeks. (C) Analysis of xenograft models deriving from sorted EGFRvIII^{High} and EGFRvIII^{Low} tumor cell populations from (A) that give rise to tumors with an EGFRvIII^{High}/EGFRvIII^{Low} tumor cell composition similar to that of initial, untreated GBM39 tumors. Values are mean $\pm$ SEM. (D and E) GBM39 tumor cells sorted for EGFRvIII expression and plated at a single cell per well and stained for EGFRvIII (red) and 4',6-diamidino-2-phenylindole (DAPI) (blue) give rise to heterogeneous colonies. (F) Identical EGFRvIII^{High}/EGFRvIII^{Low} composition from tumor cells sorted in (D) and plated at 2 to 5 cells per well. Values are mean $\pm$ SEM from $n = 5$ independent cultures.

EGFRvIII^{High} GBMs display enhanced apoptotic sensitivity to EGFR TKIs (Fig. 1F). However, our data suggest that EGFR TKI resistance may not be mediated entirely by selection for a subpopulation of tumor cells lacking extrachromosomal *EGFRvIII* DNA but may also involve rapid single-step elimination, as has been described for loss of the *DHFR* gene on double minutes in the absence of methotrexate (26, 27) and the loss of *MYCC* or *MYCN* from double minutes in the presence of hydroxyurea (28). These independent and complementary mechanisms of EGFRvIII regulation, coupled to intercellular signaling between EGFRvIII^{High}/EGFRvIII^{Low} tumor cells (6), enable GBMs to achieve an EGFRvIII^{High}/EGFRvIII^{Low} ratio that is optimal for growth and survival,

including in response to EGFR TKI therapy. Future work is needed to examine the compensatory mechanisms that enable GBM cells lacking *EGFRvIII* extrachromosomal DNA to continue to proliferate during EGFR TKI treatment, including the potential role of extrachromosomal *MDM2* amplification, and possible changes along the spectrum of epithelial mesenchymal transition, including irreversible up-regulation of ZEB-1 (fig. S14), which recently has been shown to promote plasticity and tumorigenicity of breast cancer cells (29).

Resistance to targeted therapies is a nearly universal clinical challenge for cancer patients (1, 3). Daily dosing with the EGFR TKIs is not optimal because it is hard to achieve sufficient levels of intratumoral EGFR inhibition (7). Pulsatile

intermittent treatment with much higher doses of an EGFR TKI could potentially lead to better target inhibition and even possibly less toxicity relative to continuous dosing. In other cancers, a “drug holiday” can resensitize tumors to targeted therapy (30). The data presented here provide a conceptual mechanistic rationale for pulsatile intermittent EGFR TKI dosing in GBM to achieve better target inhibition while permitting tumors to regain drug sensitivity as extrachromosomal *EGFRvIII* DNA levels rapidly rise between treatments (fig. S11). These results provide an unexpected twist showing that loss of *EGFRvIII* extrachromosomal DNA promotes resistance, in contrast to the current paradigm of drug resistance through increased levels of extrachromosomal

Fig. 3. GBM cells suppress EGFRvIII protein expression on prolonged exposure to erlotinib and up-regulate it upon drug withdrawal by restoring EGFR⁺ extrachromosomal DNA elements. (A) Schematic model of reversible EGFR TKI resistance model. GBM39 cells were maintained in neurosphere culture and were treated continuously with vehicle (naïve) or erlotinib [5 μ M, erlotinib-resistant (ER), 60 days]. Drug was removed from the ER neurospheres for 30 days [drug-removed (DR)]. (B) Immunoblot of EGFRvIII levels for naïve, ER, and DR cells. (C) MIC chip quantification of the ratio of EGFRvIII^{High} and EGFRvIII^{Low} tumor cells in naïve, ER, and DR cells. (D). DAPI-stained metaphases of naïve, ER, and DR cells probed with *EGFR* (red) and chromosome 7 centromere probes (CEP7, green) with abundant *EGFR*⁺ extrachromosomal DNA elements in naïve and DR GBM cells. No extrachromosomal *EGFR*⁺ DNA elements were detected in any of the ER metaphase spreads. The white arrow shows *EGFR*⁺ HSR-like staining of a marker chromosome lacking centromere 7. One such DNA element was found in metaphases from each ER GBM cell analyzed. They were also detected in some naïve and drug-removed metaphases. (E) Map of *EGFR* gene between exon 1 and intron 8. (F) Southern blot analysis shows binding of *EGFR* probe (red line) to low-molecular-weight DNA, which is lost during resistance and reemerges with drug withdrawal. Normal genomic DNA is used as control for *EGFR* probe. (G) PCR using primers spanning each of the 17 Bam H1 restriction sites from 5' of exon 1 through intron 8 (see supplementary materials) was used to identify EGFRvIII or wild-type *EGFR*. Primer pairs 13/17 and 14/17 span regions that are 32 kb apart in wild-type *EGFR* but only slightly more than 4 kb in EGFRvIII. Primer pairs 13/17 and 14/17 cannot amplify wild-type *EGFR* but result in amplification of EGFRvIII from low-molecular-weight DNA of naïve and drug-removed GBM39 cells. No EGFRvIII was detected in erlotinib-resistant GBM39 cells. Primers 15 and 16 are both deleted in EGFRvIII but maintained in wild-type *EGFR*. Primer pair 16/17 yields a 3.3-kb wild-type *EGFR* band in normal control DNA. Representative images of primer pairs 16/17 and 13/17 are shown. (H and I) Sequencing of the cloned fragments reveals identical intronic breakpoints associated with a 27,785–base pair deletion of exon 3 to 7 sequences and resulting in EGFRvIII transcript and protein in treatment-naïve and drug-removed GBM39 cells.

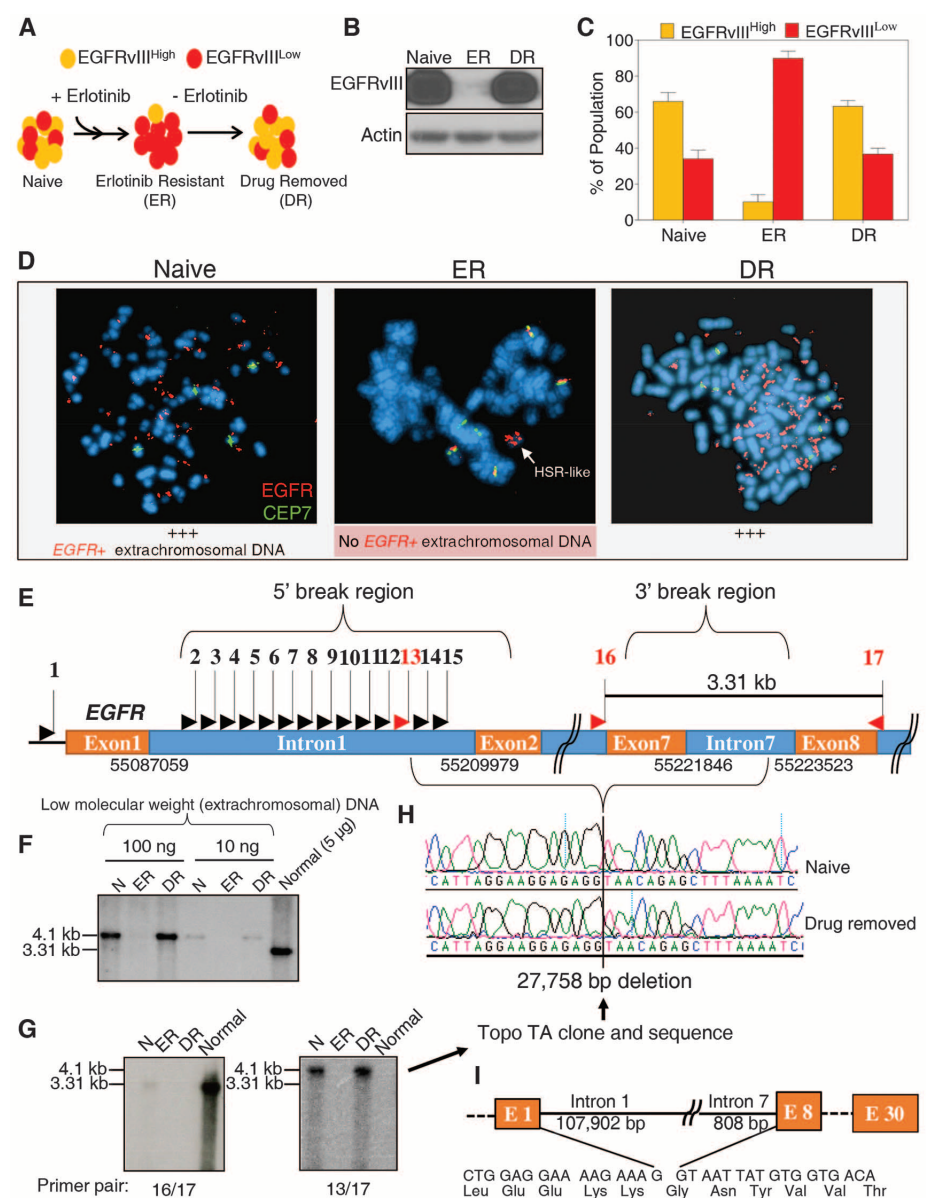
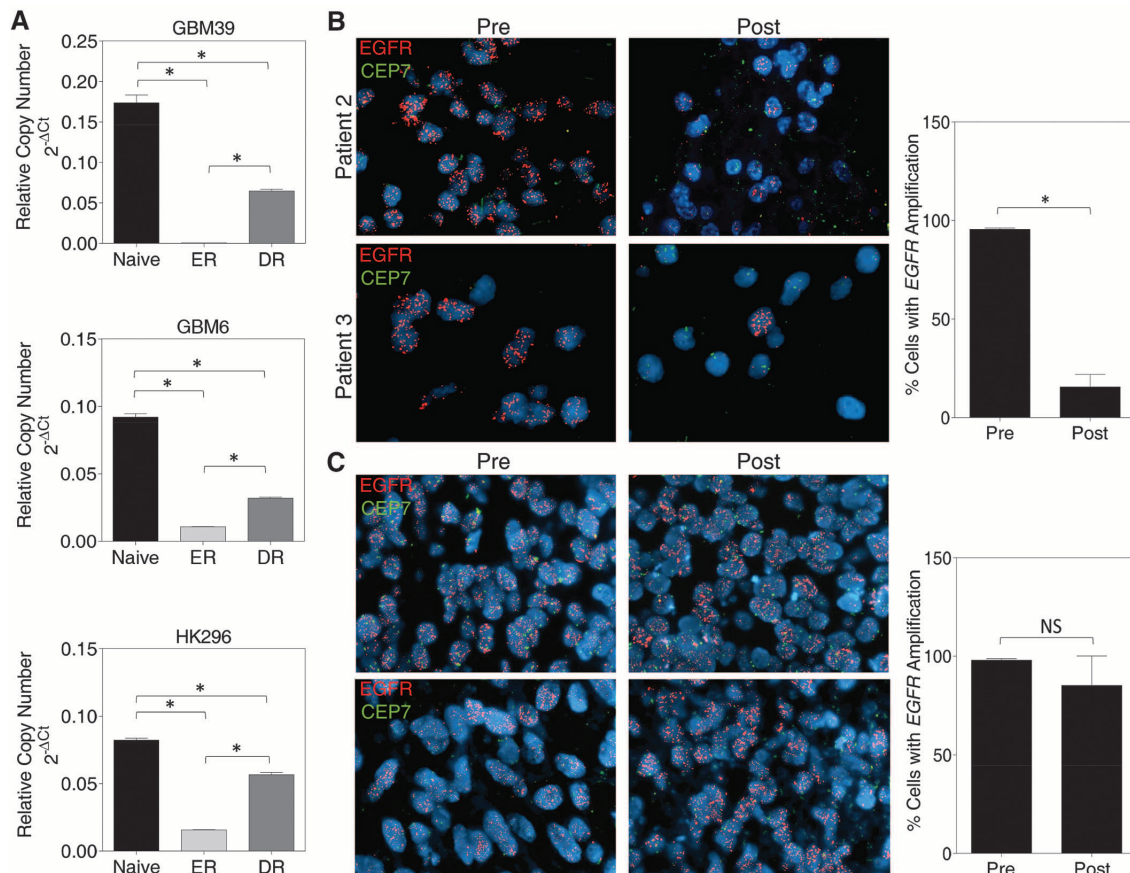


Fig. 4. Loss of *EGFR* extrachromosomal DNA elements in GBM patient samples treated with *EGFR* TKI. (A) Quantitative PCR analysis of *EGFR*VIII extrachromosomal DNA in three GBM patient-derived neurosphere lines. Erlotinib resistance for GBM6 and HK296 was established by continuous erlotinib treatment (1 μ M) for 30 days. DR for GBM6 and HK296 was established after removing erlotinib from ER cultures for 3 days. Conditions for GBM39 were described above. Values are mean $\pm$ SEM from $n > 9$ replicates. $*P < 0.0001$ from unpaired t test. (B) Representative images (left) and quantification (right) from dual-color FISH (CEP7, green; *EGFR*, red) performed on pre/post matched pairs of GBM tissue sections from $n = 2$ patients treated with lapatinib for 10 days (patients #2 and #3 from Fig. 1). Nuclei were counterstained with DAPI. Values are mean $\pm$ SD. $*P < 0.005$ from unpaired t test. (C) Representative images (left) and quantification (right) from dual-color FISH (CEP7, green; *EGFR*, red) performed on pre/post matched pairs of GBM tissue sections from $n = 4$ *EGFR*VIII-positive patients treated with radiation and concomitant chemotherapy using standard dosing of temozolomide. Nuclei were counterstained with DAPI. Values are mean $\pm$ SD.



DNA carrying drug resistance genes (31, 32). These results also highlight the diversity of mechanisms by which extrachromosomal DNA can promote resistance to targeted cancer therapies. A number of other oncogenes have been identified on extrachromosomal DNA (33), potentially enabling them to respond rapidly to targeted drug treatment (34). It is possible that resistance in other tumor types in which the main oncogene is extrachromosomal may be similarly mediated by loss of the oncogene on extrachromosomal DNA.

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Supplementary Materials

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Materials and Methods
Figs. S1 to S14
References

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A Cascade of Histone Modifications Induces Chromatin Condensation in Mitosis

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Metaphase chromosomes are visible hallmarks of mitosis, yet our understanding of their structure and of the forces shaping them is rudimentary. Phosphorylation of histone H3 serine 10 (H3 S10) by Aurora B kinase is a signature event of mitosis, but its function in chromatin condensation is unclear. Using genetically encoded ultraviolet light-inducible cross-linkers, we monitored protein-protein interactions with spatiotemporal resolution in living yeast to identify the molecular details of the pathway downstream of H3 S10 phosphorylation. This modification leads to the recruitment of the histone deacetylase Hst2p that subsequently removes an acetyl group from histone H4 lysine 16, freeing the H4 tail to interact with the surface of neighboring nucleosomes and promoting fiber condensation. This cascade of events provides a condensin-independent driving force of chromatin hypercondensation during mitosis.

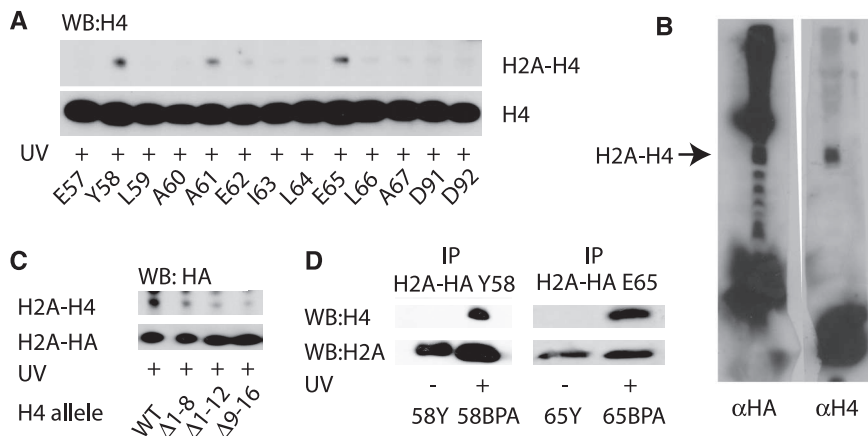
Metaphase chromosome condensation requires the condensin complex, but additional factors must exist because compaction still occurs when condensin is disrupted (1, 2). Phosphorylation of histone H3 Ser¹⁰ (H3 S10) by the kinase Aurora B—an event that correlates with mitosis in all organisms (3)—drives hypercondensation of an artificially elongated chromosome during anaphase of budding yeast (4), which suggests that histone tail modifications may influence chromosome condensation. In vitro, the flexible N-terminal domains of the core histones mediate the condensation of arrays of nucleosome core particles upon addition

of Mg²⁺ ions. Acetylation of histone H4 Lys¹⁶ (H4 K16) counteracts this process (5, 6), but phosphorylation of H3 S10 has no effect (7). The N terminus of histone H4 contacts the negatively charged, “acidic” patch formed by the H2A-H2B dimer interface on the surface of a neighboring nucleosome. This interaction is critical for the formation of higher-order chromatin structures (5, 8) and has been trapped by the formation of disulfide bridges between suitably installed cysteines (9, 10). These observations correlate with defects in the formation of heterochromatin-like structures in yeast caused by mutations in H4 K16 (11, 12). However, all evidence that this interaction mediates chromosome condensation during mitosis is indirect.

Here, we inserted the ultraviolet light (UV)-inducible cross-linker amino acid *p*-benzoyl-L-phenylalanine (pBPA) in histone proteins of living yeast, using genetic code expansion (13, 14) to trace the dynamic interactions of these proteins along the cell cycle. We expressed *Escherichia coli*

Tyr-tRNA_{CUA} and its cognate pBPA-specific tRNA synthetase in *Saccharomyces cerevisiae* cells (15), leading to the incorporation of pBPA in response to amber codons in an additional, plasmid-borne histone H2A gene [tagged with hemagglutinin (HA) and under its native promoter]. Depending on the presence of pBPA in the medium, these cells produce an additional small amount (~10%) of the histone containing pBPA at the encoded site (fig. S1). We introduced amber codons at positions 57 to 67, 91, and 92 of *S. cerevisiae* histone H2A, which are close to the H4 tail in the crystal structure (16). Cells were irradiated with UV to induce cross-link formation, giving rise to complex but reproducible banding patterns depending on the site of pBPA incorporation (fig. S2). We observed a cross-link product of ~30 kD when pBPA replaced Tyr⁵⁸ (Y58), Ala⁶¹ (A61), or Glu⁶⁵ (E65) of H2A, which would match the size of HA-tagged H2A cross-linked to H4 (asterisks in fig. S2). Such a cross-link product was readily detected with antibodies to H4 (anti-H4) in acid extracts of isolated nuclei (Fig. 1A). This band comigrated with the cross-link product detected with antibodies to HA (anti-HA) when analyzed on the same Western blot (Fig. 1B). Deletions in the H4 tail, especially when H4 K16 was affected, strongly diminished cross-linking (Figs. 1C and 2C). Finally, immunoprecipitation of the cross-link products with anti-HA revealed an H4 cross-reactive band at the expected molecular weight (Fig. 1D). Thus, these experiments identify a cross-link product between H4 and the acidic patch of H2A in vivo. Surprisingly, the H2A-H4 cross-link is of relatively low abundance compared to other cross-link products. However, cross-linking efficiency is influenced by the chemical environment and may not reflect the relative abundance of different interactions. Accordingly, reconstituted arrays containing pBPA produced the same cross-link—which depended on salt-induced array condensation—with similar efficiency (fig. S3); hence, even under optimal conditions, only a minor fraction formed cross-links.

Fig. 1. Identification of H2A-H4 cross-link products. (A) Western blot analysis (anti-H4) of acid-extracted histones from cells expressing H2A-HA with pBPA at the indicated positions. (B) H2A-HA Y58pBPA-expressing cells were cross-linked and acid-extracted histones analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). The membrane was cut in half and analyzed separately using either anti-HA or anti-H4. (C) Deletions in the H4 tail affect the interaction with the acidic patch. Yeast cells carrying genomic mutations in H4 producing H2A-HA Y58pBPA were arrested with nocodazole. Whole-cell extracts were analyzed with anti-HA. (D) Histones extracted from yeasts producing H2A-HA Y58Y, H2A-HA Y58pBPA, H2A-HA E65Y, or H2A-HA E65pBPA by amber suppression were immunoprecipitated with anti-HA under denaturing conditions and analyzed by Western blot. See figs. S2, S9, and S10 for full-sized Western blots.



The H2A-H4 interaction showed a strong dependence on cell cycle stage, with a maximum in M phase (40 to 80 min) coinciding with phosphorylation of H3 S10 (3) (Fig. 2A). H4 K16ac is predominant in S phase (20 min and 100 min) and lowest during M phase, anticorrelating with the intensity of the H2A-H4 cross-link. This indicates that the H2A-H4 interaction, modulated by the acetylation of H4 K16, may contribute to chromatin condensation *in vivo*. We observed the same cell cycle-dependent behavior of the cross-link product with anti-H4 in acid extracts of isolated nuclei (Fig. 2B).

The interaction of the H4 N terminus with the acidic patch is prevented by acetylation of Lys¹⁶ *in vitro* (5, 6). We therefore tested strains with mutations in the N-terminal tail of H4 for their ability to produce a H2A-H4 cross-link (Fig. 2C and fig. S4). H4 K16A strongly reduced cross-link formation, in agreement with its effect on the compaction of reconstituted chromatin arrays (5). H4 K16Q had a less pronounced impact, suggesting that glutamine is not a good mimic of acetyl-lysine in this context. In asynchronous H4 K16R cells, where acetylation is prevented, the interaction was not affected, but in synchronized cells we observed that the H2A-H4 cross-linking efficiency no longer cycled (Fig. 2D). This suggests that H4 K16 acetylation is a key regulator of chromatin condensation in mitosis.

H3 S10 phosphorylation by Aurora B is a marker of mitosis, but it is unknown whether and how it contributes to chromatin condensation (3). Cells with a H3 S10A mutation showed substantially reduced H2A-H4 cross-linking efficiency, whereas a phospho-mimetic S10D mutation had no effect (Fig. 3A). H4 K16 acetylation anticorrelated with S10 phosphorylation, indicating that the latter acts upstream of H4 K16 deacetylation. In whole-cell lysates of synchronized H3 S10A cells, we found that these defects persist throughout the cell cycle (Fig. 3B). We confirmed these observations by quantifying the abundance of the H2A-H4 cross-link with anti-H4 in a synchronization experiment in which we acid-extracted the histones from isolated nuclei (dotted line in Fig. 3B, lower right; Western blots in fig. S5). In H3 S10D mutants, H4 K16 reacylation was delayed after mitosis (20- to 60-min time points in Fig. 3B); fluorescence-activated cell sorter (FACS) analysis showed that these cells have difficulties exiting mitosis after release from nocodazole (fig. S6). Hence, the dynamics of these posttranslational modifications must be correctly synchronized to ensure timely formation of the H2A-H4 interaction. A T3A mutation or deletion of four or more residues from the N terminus of H3 greatly diminished H2A-H4 cross-linking efficiency, whereas a phospho-mimetic T3D mutation had no effect (Fig. 3A and fig. S7), indicating that phosphorylation of this residue is also involved in the pathway. In fission yeast and vertebrates, phosphorylation of H3 T3 by Haspin kinase recruits the chromosomal passenger complex (CPC), including Aurora B, to the H3 tail to phosphorylate

H3 S10 (17–19). Indeed, deletion of both Haspin kinase homologs (*alk1/2Δ*) attenuated H3 S10 phosphorylation and produced the same downstream effects as a H3 S10A mutation (Fig. 3A).

These findings imply that phosphorylated H3 mediates recruitment of a H4 K16 deacetylase. Indeed, strains deleted for the *SIR2* homolog *HST2* were unable to deacetylate H4 K16 and to form the H2A-H4 interaction, although H3 S10 phosphorylation was unaffected (Fig. 3A). Neither H4 K16ac nor the H2A-H4 interaction showed any dependence on the cell cycle in the absence of Hst2p (Fig. 3B). Hence, H3 S10 phosphorylation is required, directly or indirectly, to recruit Hst2p to condensing chromatin. Accordingly, pull-down experiments with synthetic H3 peptides phosphorylated at Ser¹⁰ efficiently recovered Hst2p but not Sir2p from whole-cell extracts, relative to a nonphosphorylated control (Fig. 3C).

To test whether Hst2p and H4 K16 deacetylation stimulate chromatin condensation in mitosis, we asked whether Hst2p supports the condensation of the long compound chromosome *LC(XII:IV) cen12Δ*, which hypercondenses in anaphase in an

Aurora B- and H3 S10-dependent manner (4). Hypercondensation is detected by the shortening of the distance between the fluorescently tagged loci *TRP1* and *LYS4* (Fig. 4A) on *LC(XII:IV) cen12Δ* during late anaphase. Both *HST2* inactivation and the H3 S10A mutation impaired this process to the same extent, either alone or in combination (Fig. 4B). Thus, Hst2p indeed acts in the same pathway as H3 S10 to promote chromosome condensation during mitosis.

Taken together, our results indicate that during early mitosis, phosphorylation of H3 T3 by Haspin and subsequently of H3 S10 by CPC controls the recruitment of the deacetylase Hst2p to nucleosomes and the deacetylation of Lys¹⁶ on H4. As a consequence, the tail of H4 starts interacting with neighboring nucleosomes, promoting chromatin condensation (fig. S8). The recruitment of the CPC by Haspin kinase phosphorylation of H3 T3 has been demonstrated in other organisms (17–19). CPC localization to centromeres was recently shown to be independent of Haspin in *S. cerevisiae* (20). This may indicate that in this organism, different recruitment mechanisms exist between centromeres and chromosome arms.

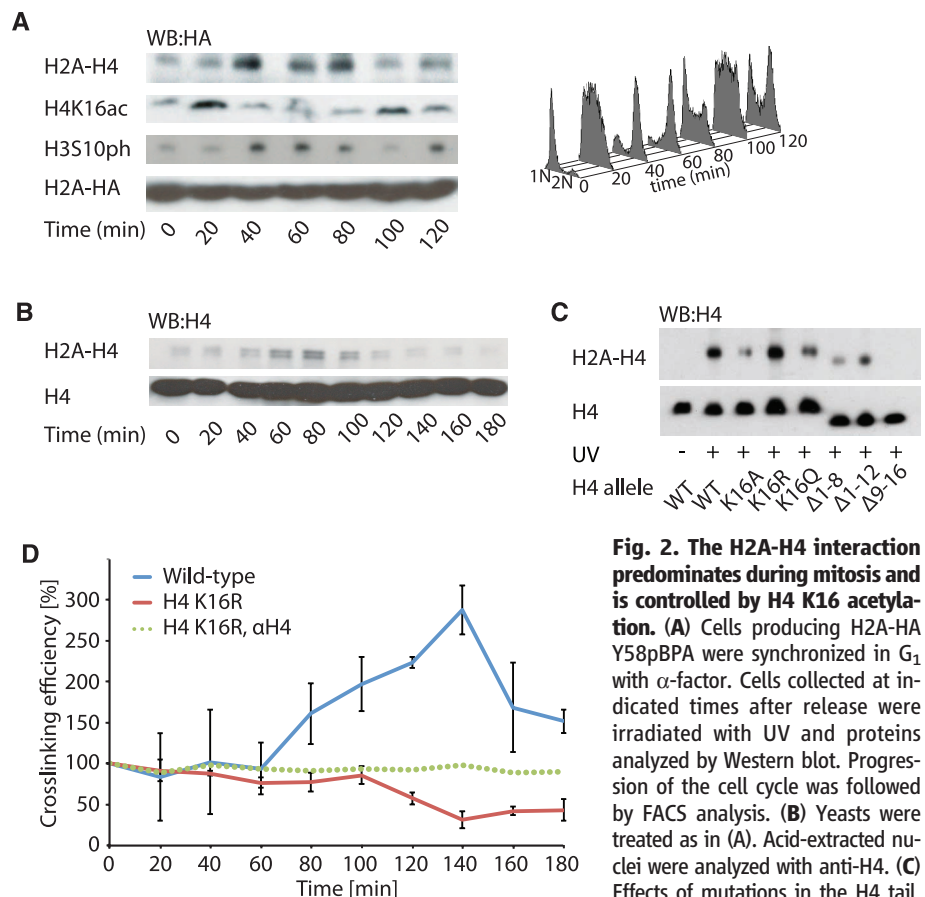


Fig. 2. The H2A-H4 interaction predominates during mitosis and is controlled by H4 K16 acetylation. (A) Cells producing H2A-HA Y58pBPA were synchronized in G₁ with α -factor. Cells collected at indicated times after release were irradiated with UV and proteins analyzed by Western blot. Progression of the cell cycle was followed by FACS analysis. (B) Yeasts were treated as in (A). Acid-extracted nuclei were analyzed with anti-H4. (C) Effects of mutations in the H4 tail. Yeast cells were treated as in Fig. 1C, and acid-extracted nuclei were analyzed with anti-H4. (D) Nocodazole synchronization of wild-type or H4 K16R mutant yeasts producing H2A-HA Y58pBPA. Cross-linking efficiency was analyzed as in (A) and quantified as percentage of the signal at $t = 0$. An identical analysis of acid-extracted nuclei that used anti-H4 to detect the H2A-H4 cross-link is shown by a dotted line. Data are means $\pm$ SD ($n = 3$). See figs. S11 to S14 for full-size Western blots and FACS analysis.

Unlike in most other organisms (21–23), mutation of H3 S10 does not cause obvious growth defects in yeast (24), but it impairs the ability of fitting the condensation state of chromosomes to spindle length through “adaptive hypercondensation” (4). Because budding yeast chromosomes are short, this process is probably not essential for chromosome segregation during most mitoses.

The pathway described here is likely to be more important in higher eukaryotes. Condensation of chromosomes in the absence of condensin has been observed in many different cell types. For example, chicken DT40 cells lacking condensin are able to condense chromatin by an activity termed “regulator of chromosome architecture”

Fig. 3. Phosphorylation of H3 S10 recruits Hst2p to deacetylate H4 K16 and induce the H2A-H4 interaction.

(A) Yeasts mutated in H3 and producing H2A-HA Y58pBPA were arrested with nocodazole and irradiated with UV; acid extracts of isolated nuclei were analyzed by Western blot with the indicated antibodies. (B) Mutant yeasts producing H2A-HA Y58pBPA were synchronized with nocodazole and treated as in Fig. 1C. An identical analysis of acid-extracted nuclei that used anti-H4 to detect the H2A-H4 cross-link is shown by a dotted line in the graph at lower right (Western blots in fig. S5). Data are means $\pm$ SD ($n = 3$). (C) Whole-cell extracts from BY4741 cells genomically tagged with green fluorescent protein (GFP) were incubated with immobilized H3 peptides with or without phosphorylated S10. Eluates were analyzed by Western blot using anti-GFP.

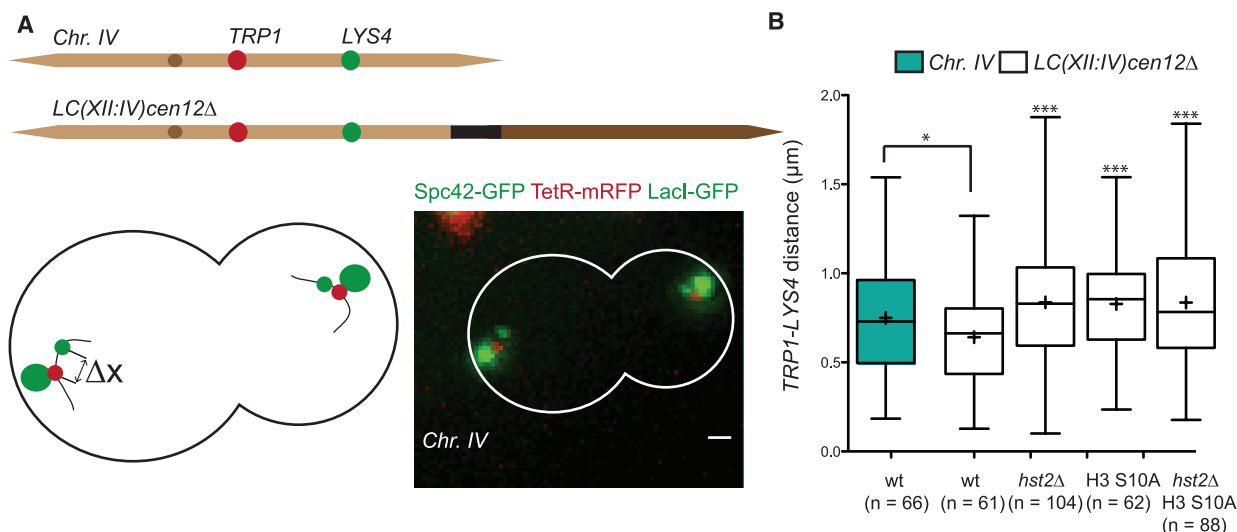
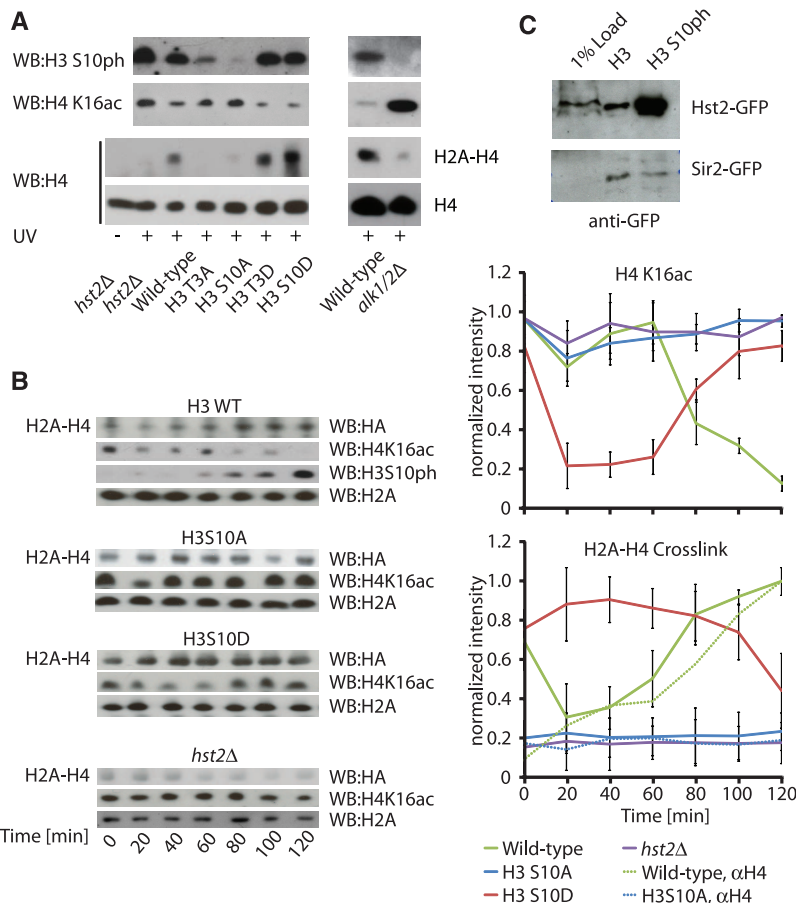


Fig. 4. Phosphorylated H3 S10 and deacetylase Hst2p act in the same pathway to hypercondense long chromosomes. (A) Schematic overview of the wild-type (Chr. IV) and long chromosome (LC(XII:IV)cen12Δ). A representative anaphase cell contains labeled SPBs (Spc42-GFP; large green dot) and TRP1 and LYS4 loci (smaller red and green dots, respectively). Distance

between the latter dots (Δx) was determined. (B) TRP1-LYS4 distance in yeast mother cells during anaphase for strains of the indicated genotype and containing the indicated chromosome conformation. Boxes include 50% of data points, whiskers 100%. Medians (lines) and means (plus signs) are shown. * $P < 0.05$, *** $P < 0.001$.

(RCA), which is negatively regulated by protein phosphatase 1 (PP1) (25). We postulate that RCA entails the interaction of the H4 tail with neighboring nucleosomes and that PP1 counteracts this process by dephosphorylating H3 S10, a known substrate of this enzyme (26). We therefore propose that, together with condensin, the signaling cascade we identified is a conserved mechanism for shaping mitotic chromosomes.

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Supplementary Materials

www.sciencemag.org/content/343/6166/77/suppl/DC1
Materials and Methods
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Genetic Screens in Human Cells Using the CRISPR-Cas9 System

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The bacterial clustered regularly interspaced short palindromic repeats (CRISPR)–Cas9 system for genome editing has greatly expanded the toolbox for mammalian genetics, enabling the rapid generation of isogenic cell lines and mice with modified alleles. Here, we describe a pooled, loss-of-function genetic screening approach suitable for both positive and negative selection that uses a genome-scale lentiviral single-guide RNA (sgRNA) library. sgRNA expression cassettes were stably integrated into the genome, which enabled a complex mutant pool to be tracked by massively parallel sequencing. We used a library containing 73,000 sgRNAs to generate knockout collections and performed screens in two human cell lines. A screen for resistance to the nucleotide analog 6-thioguanine identified all expected members of the DNA mismatch repair pathway, whereas another for the DNA topoisomerase II (*TOP2A*) poison etoposide identified *TOP2A*, as expected, and also cyclin-dependent kinase 6, *CDK6*. A negative selection screen for essential genes identified numerous gene sets corresponding to fundamental processes. Last, we show that sgRNA efficiency is associated with specific sequence motifs, enabling the prediction of more effective sgRNAs. Collectively, these results establish Cas9/sgRNA screens as a powerful tool for systematic genetic analysis in mammalian cells.

A critical need in biology is the ability to efficiently identify the set of genes underlying a cellular process. In microorganisms, powerful methods allow systematic loss-of-function genetic screening (1, 2). In mammalian cells, however, current screening methods fall short—

primarily because of the difficulty of inactivating both copies of a gene in a diploid mammalian cell. Insertional mutagenesis screens in cell lines that are near-haploid or carry *Blm* mutations, which cause frequent somatic crossing-over, have proven powerful but are not applicable to most cell lines and suffer from integration biases of the insertion vectors (3, 4). The primary solution has been to target mRNAs with RNA interference (RNAi) (5–9). However, this approach is also imperfect because it only partially suppresses target gene levels and can have off-target effects on other mRNAs, resulting in false negative and false positive results (10–12). Thus, there remains an unmet need for an efficient, large-scale, loss-of-function screening method in mammalian cells.

Recently, the clustered regularly interspaced short palindromic repeats (CRISPR) pathway, which functions as an adaptive immune system in bacteria (13), has been co-opted to engineer mammalian genomes in an efficient manner (14–16). In this two-component system, a single-guide RNA (sgRNA) directs the Cas9 nuclease to cause double-stranded cleavage of matching target DNA sequences (17). In contrast to previous genome-editing techniques, such as zinc-finger nucleases and transcription activator-like effector nucleases (TALENs), the target specificity of CRISPR-Cas9 is dictated by a 20-base pair (bp) sequence at the 5' end of the sgRNA, allowing for much greater ease of construction of knockout reagents. Mutant cell lines and mice bearing multiple modified alleles can be generated with this technology (18, 19).

We set out to explore the feasibility of using the CRISPR-Cas9 system to perform large-scale, loss-of-function screens in mammalian cells. The idea was to use a pool of sgRNA-expressing lentivirus to generate a library of knockout cells that could be screened under both positive and negative selection. Each sgRNA would serve as a distinct DNA barcode that can be used to count the number of cells carrying it by using high-throughput sequencing (Fig. 1A). Pooled screening requires that single-copy sgRNA integrants are sufficient to induce efficient cleavage of both copies of a targeted locus. This contrasts with the high expression of sgRNAs achieved through transfection that is typically used to engineer a specific genomic change by using the CRISPR-Cas9 system.

We first tested the concept in the near-haploid, human KBM7 CML cell line by creating a clonal derivative expressing the Cas9 nuclease (with a FLAG-tag at its N terminus) under a doxycycline-inducible promoter (Fig. 1B). Transduction of these cells at low multiplicity of infection (MOI) with a lentivirus expressing a sgRNA targeting the endogenous AAVS1 locus revealed substan-

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tial cleavage at the AAVS1 locus 48 hours after infection (Fig. 1C). Moreover, because the sgRNA was stably expressed, genomic cleavage continued to increase over the course of the experiment. Deep sequencing of the locus revealed that repair of Cas9-induced double-strand breaks resulted in small deletions (<20 bp) in the target sequence, with tiny insertions or substitutions (<3 bp) occurring at a lower frequency (Fig. 1D). The vast majority of the lesions, occurring in a protein-coding region, would be predicted to give rise to a nonfunctional protein product, indicating that CRISPR-Cas9 is an efficient means of generating loss-of-function alleles.

We also analyzed off-target activity of CRISPR-Cas9. Although the specificity of CRISPR-Cas9 has been extensively characterized in transfection-based settings (20–22), we wanted to examine its off-target behavior in our system, in which Cas9 and a sgRNA targeting AAVS1 (sgAAVS1) were stably expressed for 2 weeks. We compared the level of cleavage observed at the target locus (97%) with levels at 13 potential off-target cleavage sites in the genome (defined as sites differing by up to 3 bp from sgAAVS1) (fig. S1A). Minimal cleavage (<2.5%) was observed at all sites,

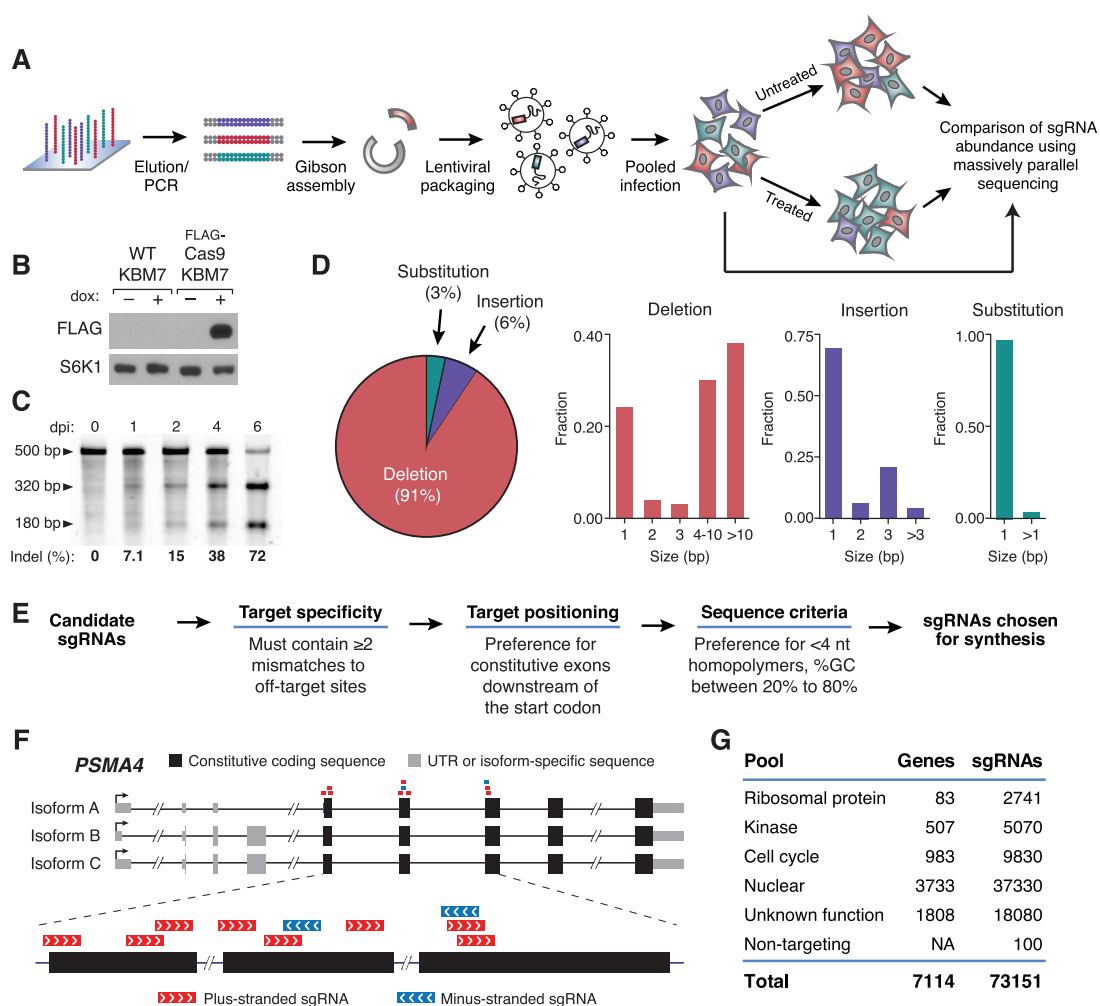
with one exception, which was the only site that had perfect complementarity in the “seed” region (terminal 8 bp) (fig. S1B). On average, sgRNAs have ~2.2 such sites in the genome, almost always (as in this case) occurring in noncoding DNA and thus less likely to affect gene function (supplementary text S1).

To test the ability to simultaneously screen tens of thousands of sgRNAs, we designed a sgRNA library with 73,151 members, consisting of multiple sgRNAs targeting 7114 genes and 100 nontargeting controls (Fig. 1E, table S1, and supplementary materials, materials and methods). sgRNAs were designed against constitutive coding exons near the beginning of each gene and filtered for potential off-target effects based on sequence similarity to the rest of the human genome (Fig. 1, F and G). The library included 10 sgRNAs for each of 7031 genes and all possible sgRNAs for each of the 83 genes encoding ribosomal proteins (Fig. 1H). To assess the effective representation of our microarray synthesized library, we sequenced sgRNA barcodes from KBM7 cells 24 hours after infection with the entire lentiviral pool and were able to detect the overwhelming majority (>99%) of our sgRNAs, with high uniformity across con-

structs (only a sixfold increase in abundance between the 10th and 90th percentiles) (fig. S2A).

As an initial test of our approach, we screened the library for genes that function in DNA mismatch repair (MMR). In the presence of the nucleotide analog 6-thioguanine (6-TG), MMR-proficient cells are unable to repair 6-TG-induced lesions and arrest at the G2-M cell-cycle checkpoint, whereas MMR-defective cells do not recognize the lesions and continue to divide (23). We infected Cas9-KBM7 cells with the entire sgRNA library, cultured the cells in a concentration of 6-TG that is lethal to wild-type (WT) KBM7 cells, and sequenced the sgRNA barcodes in the final population. sgRNAs targeting the genes encoding the four components of the MMR pathway (*MSH2*, *MSH6*, *MLH1*, and *PMS2*) (24) were dramatically enriched in the 6-TG-treated cells. At least four independent sgRNAs for each gene showed very strong enrichment, and barcodes corresponding to these genes made up >30% of all barcodes (Fig. 2, A and B). Each of the 20 most abundant sgRNAs targeted one of these four genes. The fact that few of the other 73,000 sgRNAs scored highly in this assay suggests a low frequency of off-target effects.

Fig. 1. A pooled approach for genetic screening in mammalian cells by using a lentiviral CRISPR-Cas9 system. (A) Outline of sgRNA library construction and genetic screening strategy. **(B)** Immunoblot analysis of WT KBM7 cells and KBM7 cells transduced with a doxycycline-inducible FLAG-Cas9 construct upon doxycycline induction. S6K1 was used as a loading control. **(C)** Sufficiency of single-copy sgRNAs to induce genomic cleavage. Cas9-expressing KBM7 cells were transduced with AAVS1-targeting sgRNA lentivirus at low MOI. The SURVEYOR mutation detection assay was performed on cells at the indicated days post-infection (dpi). Briefly, mutations resulting from cleavage of the AAVS1 locus were detected through polymerase chain reaction (PCR) amplification of a 500-bp amplicon flanking the target sequence, re-annealing of the PCR product, and selective digestion of mismatched heteroduplex fragments. **(D)** Characterization of mutations induced by CRISPR-Cas9 as analyzed with high-throughput sequencing. **(E)** sgRNA library design pipeline. **(F)** Example of sgRNAs designed for *PSMA4*. sgRNAs targeting constitutive exonic coding sequences nearest to the start codon were chosen for construction. **(G)** Composition of genome-scale sgRNA library.



We next addressed the challenge of loss-of-function screening in diploid cells, which require biallelic inactivation of a target gene. We therefore generated an inducible Cas9 derivative of the HL60 pseudo-diploid human leukemic cell line. In both HL60 and KBM7 cells, we screened for genes whose loss conferred resistance to etoposide, a chemotherapeutic agent that poisons DNA topoisomerase IIA (*TOP2A*). To identify hit genes, we calculated the difference in abundance between the treated and untreated populations for each sgRNA, calculated a score for each gene using a Kolmogorov-Smirnov test to compare the sgRNAs targeting the gene against the nontargeting control sgRNAs, and corrected for multiple hypothesis testing (Fig. 2, C to E, and table S2). Identical genes were detected in both screens, with significance levels exceeding all other genes by more than 100-fold. As expected, loss of *TOP2A* itself conferred strong protection to etoposide (25). The screen also revealed a role for *CDK6*, a G₁ cyclin-dependent kinase, in mediating etoposide-induced cytotoxicity. Every one of the 20 sgRNAs in the library targeting *TOP2A* or *CDK6* was strongly enriched (>90th percentile) in both screens, indicating that the effective coverage of our libraries is very high. We generated isogenic HL60 cell lines with individual sgRNAs against *TOP2A* and *CDK6* and, consistent with the screen results, these lines were much more resistant to etoposide

than parental or sgAAVS1-modified HL60 cells (Fig. 2, F and G). Thus, our Cas9/sbRNA system enables large-scale positive selection loss-of-function screens.

To identify genes required for cellular proliferation, we screened for genes whose loss conferred a selective disadvantage on cells. Such a screen requires accurate identification of sgRNAs that are depleted from the final cell population. A sgRNA will show depletion only if cleavage of the target gene occurs in the majority of cells carrying the construct.

As an initial test, we screened KBM7 cells with a small library containing sgRNAs targeting the *BCR* and *ABL1* genes (table S3). The survival of KBM7 cells depends on the fusion protein produced by the BCR-ABL translocation (26). As expected, depletion was seen only for sgRNAs targeting the exons of *BCR* and *ABL1* that encode the fusion protein, but not for those targeting the other exons of *BCR* and *ABL1* (Fig. 3A).

We then infected Cas9-HL60, Cas9-KBM7, and WT KBM7 cells with the entire 73,000-member sgRNA library and used deep sequencing of the sgRNA barcodes to monitor the change in abundance of each sgRNA between the initial seeding and a final population obtained after 12 cell doublings (fig. S2, A and B).

We began by analyzing ribosomal protein genes, for which the library contained all possible

sgRNAs. We observed strong Cas9-dependent depletion of sgRNAs targeting genes encoding ribosomal proteins, with good concordance between the sets of ribosomal protein genes essential for cell proliferation in the HL60 and KBM7 screens (the median sgRNA fold-change in abundance was used as a measure of gene essentiality) (Fig. 3, B and C). A few ribosomal protein genes were not found to be essential. These were two genes encoded on chromosome Y [*RPS4Y2*, which is testes-specific (27), and *RPS4Y1*, which is expressed at low levels as compared with its homolog *RPS4X* on chromosome X (28)] and “ribosome-like” proteins, which may be required only in select tissues (27) and generally are lowly expressed in KBM7 cells (fig. S3A).

We then turned our attention to other genes within our data set, for which 10 sgRNAs were designed. As for the ribosomal genes, the essentiality scores of these genes were also strongly correlated between the two cell lines (fig. S3B and table S4). For the 20 highest scoring genes, we found independent evidence for essentiality, based primarily on data from large-scale functional studies in model organisms (table S5).

To evaluate the results at a global level, we tested 4722 gene sets to see whether they showed strong signatures of essentiality by using gene set enrichment analysis (29). Gene sets related to fundamental biological processes—including

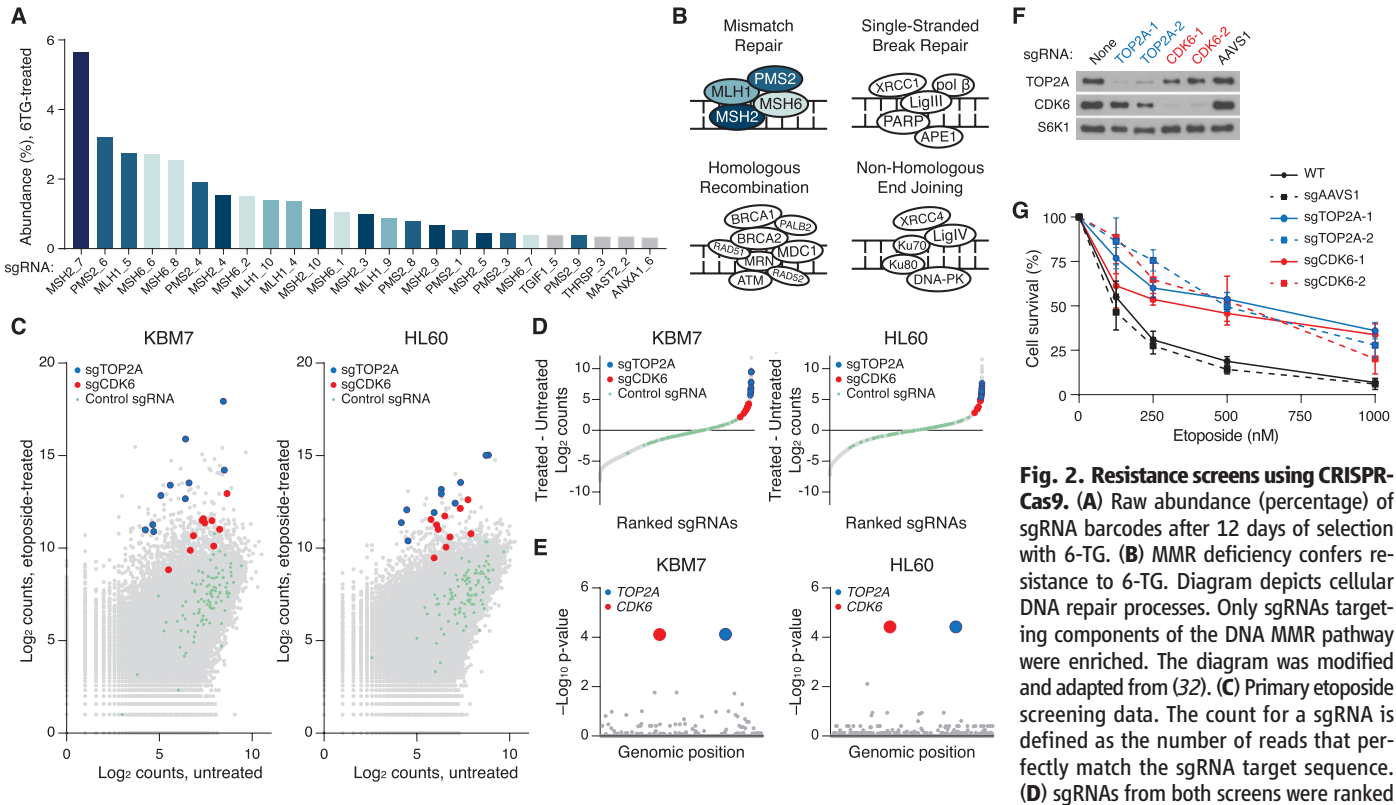


Fig. 2. Resistance screens using CRISPR-Cas9. (A) Raw abundance (percentage) of sgRNA barcodes after 12 days of selection with 6-TG. (B) MMR deficiency confers resistance to 6-TG. Diagram depicts cellular DNA repair processes. Only sgRNAs targeting components of the DNA MMR pathway were enriched. The diagram was modified and adapted from (32). (C) Primary etoposide screening data. The count for a sgRNA is defined as the number of reads that perfectly match the sgRNA target sequence. (D) sgRNAs from both screens were ranked

by their differential abundance between the treated versus untreated populations. For clarity, sgRNAs with no change in abundance are omitted. (E) Gene hit identification by comparing differential abundances of all sgRNAs targeting a gene with differential abundances of nontargeting sgRNAs in a one-sided Kolmogorov-Smirnov test. *P* values are corrected for multiple hypothesis testing. (F) Immunoblot analysis of WT and sgRNA-modified HL60 cells 1 week after infection. S6K1 was used as a loading control. (G) Viability, as measured by cellular ATP concentration, of WT and sgRNA-modified HL60 cells at indicated etoposide concentrations. Error bars denote SD (*n* = 3 experiments per group).

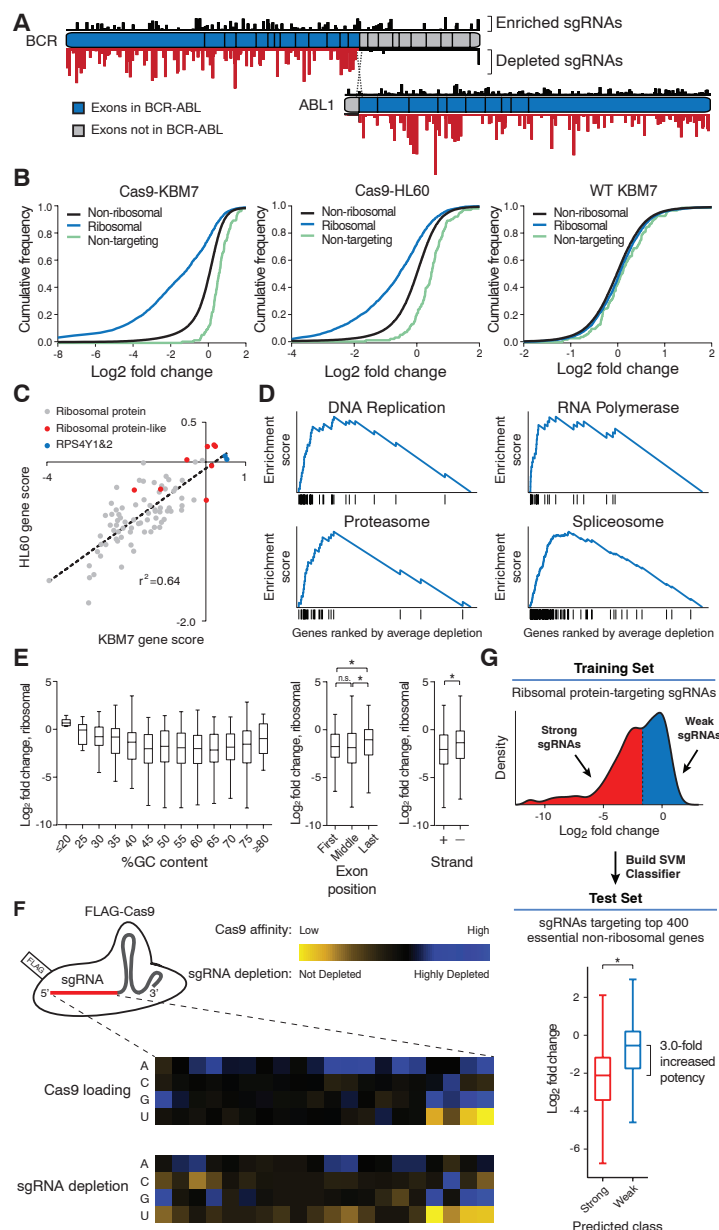


Fig. 3. Negative selection screens using CRISPR-Cas9 reveal rules governing sgRNA efficacy.

(A) Selective depletion of sgRNAs targeting exons of *BCR* and *ABL1* present in the fusion protein. Individual sgRNAs are plotted according to their target sequence position along each gene, and the height of each bar indicates the level of depletion observed. Boxes indicate individual exons. (B) Cas9-dependent depletion of sgRNAs targeting ribosomal proteins. Cumulative distribution function plots of $\log_2$ fold changes in sgRNA abundance before and after 12 cell doublings in Cas9-KBM7, Cas9-HL60, and WT-KBM7 cells. (C) Requirement of similar sets of ribosomal protein genes for proliferation in the HL60 and KBM7 cells. Gene scores are defined as the median $\log_2$ fold change of all sgRNAs targeting a gene. (D) Depleted sgRNAs target genes involved in fundamental biological processes. Gene set enrichment analysis was performed on genes ranked by their combined depletion scores from screens in HL60 and KBM7 cells. Vertical lines underneath the x axis denote members of the gene set analyzed. (E) Features influencing sgRNA efficacy. Depletion ($\log_2$ fold change) of sgRNAs targeting ribosomal protein genes was used as an indicator of sgRNA efficacy. Correlation between $\log_2$ fold changes and spacer %GC content (left), exon position targeted (middle), and strand targeted (right) are depicted ($^*P < 0.05$). (F) sgRNA target sequence preferences for Cas9 loading and cleavage efficiency. Position-specific nucleotide preferences for Cas9 loading are determined by counting sgRNAs bound to Cas9 normalized to the number of corresponding genomic integrations. Heatmaps depict sequence-dependent variation in Cas9 loading (top) and ribosomal protein gene-targeting sgRNA depletion (bottom). The color scale represents the median value (of Cas9 affinity or $\log_2$ fold change) for all sgRNAs with the specified nucleotide at the specified position. (G) sgRNA efficacy prediction. Ribosomal protein gene-targeting sgRNAs were designated as “weak” or “strong” on the basis of their $\log_2$ fold change and used to train a support-vector-machine (SVM) classifier. As an independent test, the SVM was used to predict the efficacy of sgRNAs targeting 400 essential nonribosomal genes ($^*P < 0.05$).

DNA replication, gene transcription, and protein degradation—showed strong depletion, which is consistent with their essentiality (Fig. 3D and table S6).

Last, we sought to understand the features underlying sgRNA efficacy. Although the vast majority of sgRNAs against ribosomal protein genes showed depletion, detailed comparison of sgRNAs targeting the same gene revealed substantial variation in the precise amounts of depletion. These differences are unlikely to be caused by local accessibility to the Cas9/sgRNA complex inasmuch as comparable variability was observed even among sgRNAs targeting neighboring target sites of a given gene (fig. S4A). Given that our library includes all possible sgRNAs against each of the 84 ribosomal genes, the data allowed us to search for factors that might explain the differential efficacy of sgRNAs. Because the majority of ribosomal protein genes are essential, we reasoned that the level of depletion of a given ribosomal protein-targeting sgRNA could serve as a proxy for its cleavage efficiency. Applying this approach, we found several trends related to sgRNA efficacy: (i) Single-guide sequences with very high or low GC content were less effective against their targets. (ii) sgRNAs targeting the last coding exon were less effective than those targeting earlier exons, which is consistent with the notion that disruption of the terminal exon would be expected to have less impact on gene function. (iii) sgRNAs targeting the transcribed strand were less effective than those targeting the nontranscribed strand (Fig. 3E). Although these trends were statistically significant, they explained only a small proportion of differences in sgRNA efficacy (table S7).

We hypothesized that differences in sgRNA efficacy might also result from sequence features governing interactions with Cas9. To test this, we developed a method to profile the sgRNAs directly bound to Cas9 in a highly parallel manner (supplementary materials, materials and methods). By comparing the abundance of sgRNAs bound to Cas9 relative to the abundance of their corresponding genomic integrants, we found that the nucleotide composition near the 3' end of the spacer sequence was the most important determinant of Cas9 loading (Fig. 3F). Specifically, Cas9 preferentially bound sgRNAs containing purines in the last four nucleotides of the spacer sequence, whereas pyrimidines were disfavored. A similar pattern emerged when we examined depletion of ribosomal protein-targeting sgRNAs [correlation coefficient (r) = 0.81], suggesting that, in significant part, the cleavage efficiency of a sgRNA was determined by its affinity for Cas9 (table S7).

We then sought to build an algorithm to discriminate between strong and weak sgRNAs (Fig. 3G). We trained a support-vector-machine classifier based on the target sequences and depletion scores of ribosomal protein-targeting sgRNAs. As an independent test, we used the classifier to predict the efficacy of sgRNAs targeting the 400 top scoring (essential) nonribosomal

genes. The top two thirds of our predictions exhibited threefold higher efficacy than that of the remaining fraction, confirming the accuracy of the algorithm.

Using this algorithm, we designed a whole-genome sgRNA library consisting of sequences predicted to have higher efficacy (table S8). As with the sgRNA pool used in our screens, this new collection was also filtered for potential off-target matches. This reference set of sgRNAs may be useful both for targeting single genes as well as large-scale sgRNA screening.

Taken together, these results demonstrate the utility of CRISPR-Cas9 for conducting large-scale genetic screens in mammalian cells. On the basis of our initial experiments, this system appears to offer several powerful features that together provide substantial advantages over current functional screening methods.

First, CRISPR-Cas9 inactivates genes at the DNA level, making it possible to study phenotypes that require a complete loss of gene function to be elicited. In addition, the system should also enable functional interrogation of nontranscribed elements, which are inaccessible by means of RNAi.

Second, a large proportion of sgRNAs successfully generate mutations at their target sites. Although this parameter is difficult to directly assess in pooled screens, we can obtain an estimate by examining the “hit rate” at known genes. Applying a *z* score analysis of our positive selection screens, we found that over 75% (46 of 60) of sgRNAs score at a significance threshold that perfectly separates true and false positives on a gene level (fig. S5, A to D). Together, these results show that the effective coverage of our library is very high and that the rate of false negatives should be low, even in a large-scale screen.

Third, off-target effects do not appear to seriously hamper our screens, according to several lines of evidence. Direct sequencing of potential off-target loci detected minimal cleavage at secondary sites, which typically reside in noncoding regions and do not affect gene function. Moreover, in the 6-TG screens the 20 most abundant sgRNAs all targeted one of the four members of the MMR pathway. In total, they represented over 30% of the final pool, which is a fraction greater than the next 400 sgRNAs combined. In the etoposide screen, the two top genes scored far above background levels (*P* values 100-fold smaller than that of the next best gene), enabling clear discrimination between true and false-positive hits. Last, new versions of the CRISPR-Cas9 system have recently been developed that substantially decrease off-target activity (30, 31).

Although we limited our investigation to proliferation-based phenotypes, our approach can be applied to a much wider range of biological phenomena. With appropriate sgRNA libraries, the method should enable genetic analyses of mammalian cells to be conducted with a degree of rigor and completeness currently possible only in the study of microorganisms.

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Supplementary Materials

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Genome-Scale CRISPR-Cas9 Knockout Screening in Human Cells

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The simplicity of programming the CRISPR (clustered regularly interspaced short palindromic repeats)–associated nuclease Cas9 to modify specific genomic loci suggests a new way to interrogate gene function on a genome-wide scale. We show that lentiviral delivery of a genome-scale CRISPR-Cas9 knockout (GeCKO) library targeting 18,080 genes with 64,751 unique guide sequences enables both negative and positive selection screening in human cells. First, we used the GeCKO library to identify genes essential for cell viability in cancer and pluripotent stem cells. Next, in a melanoma model, we screened for genes whose loss is involved in resistance to vemurafenib, a therapeutic RAF inhibitor. Our highest-ranking candidates include previously validated genes *NF1* and *MED12*, as well as novel hits *NF2*, *CUL3*, *TADA2B*, and *TADA1*. We observe a high level of consistency between independent guide RNAs targeting the same gene and a high rate of hit confirmation, demonstrating the promise of genome-scale screening with Cas9.

A major goal since the completion of the Human Genome Project is the functional characterization of all annotated genetic elements in normal biological processes and dis-

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ease (1). Genome-scale loss-of-function screens have provided a wealth of information in diverse model systems (2–5). In mammalian cells, RNA interference (RNAi) is the predominant method for genome-wide loss-of-function screening (2, 3), but its utility is limited by the inherent incompleteness of protein depletion by RNAi and confounding off-target effects (6, 7).

The RNA-guided CRISPR (clustered regularly interspaced short palindrome repeats)–associated nuclease Cas9 provides an effective means of introducing targeted loss-of-function mutations at specific sites in the genome (8, 9). Cas9 can be programmed to induce DNA double-strand breaks

(DSBs) at specific genomic loci (8, 9) through a synthetic single-guide RNA (sgRNA) (10), which when targeted to coding regions of genes can create frame shift insertion/deletion (indel) mutations that result in a loss-of-function allele. Because the targeting specificity of Cas9 is conferred by short guide sequences, which can be easily generated at large scale by array-based oligonucleotide library synthesis (11), we sought to explore the potential of Cas9 for pooled genome-scale functional screening.

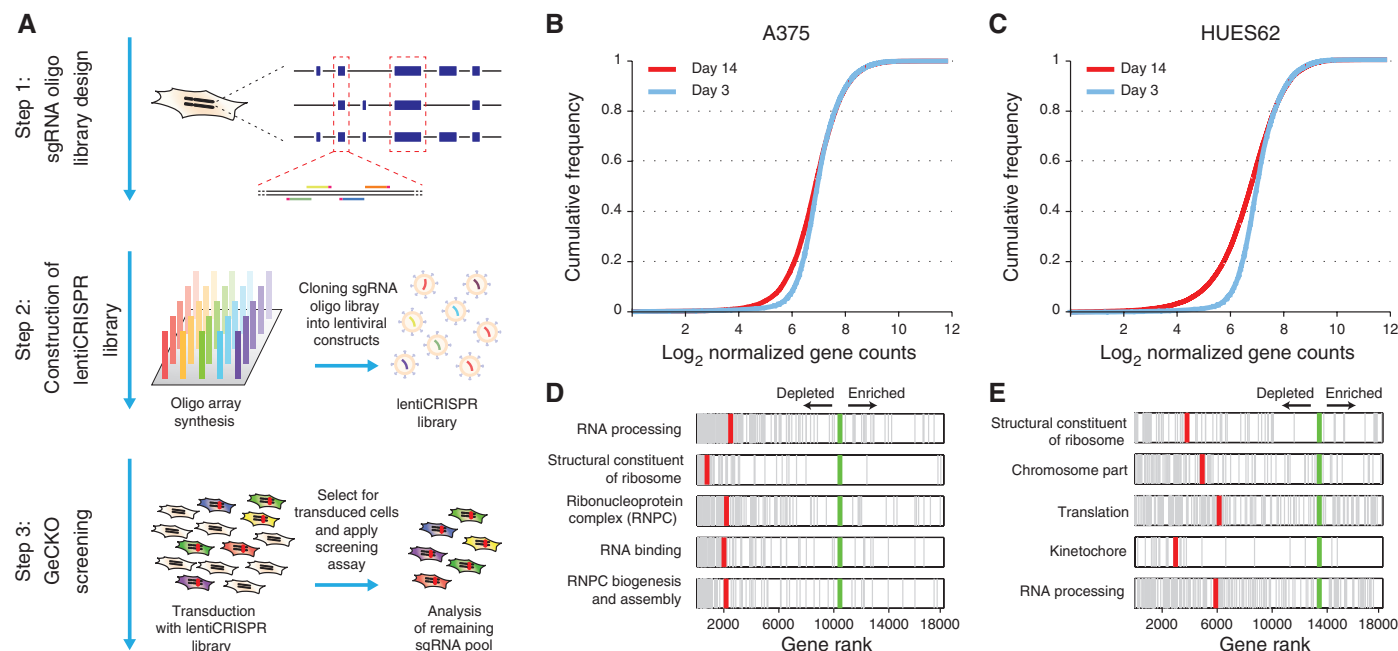
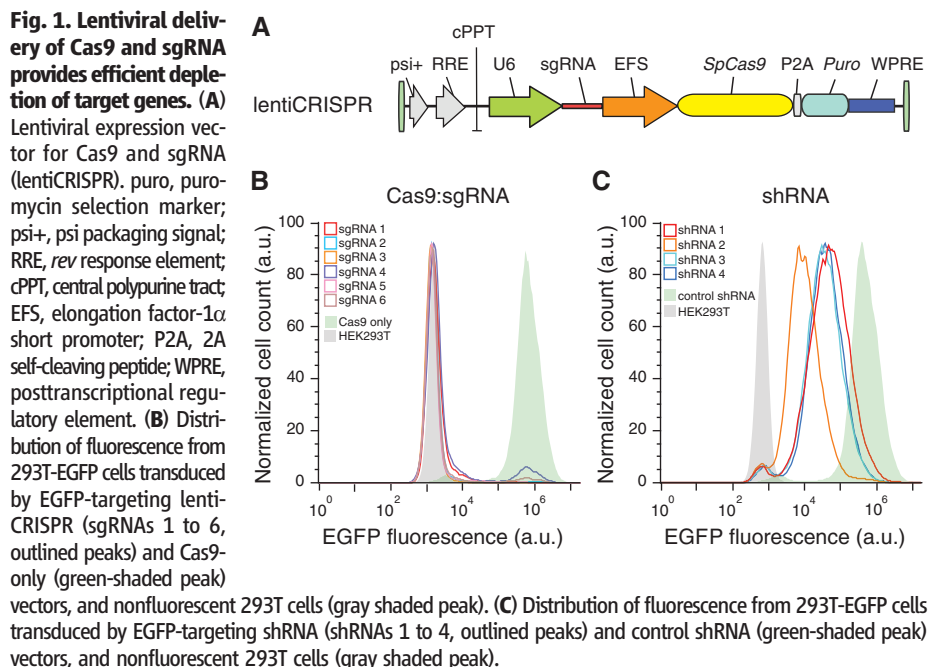
Lentiviral vectors are commonly used for delivery of pooled short-hairpin RNAs (shRNAs) in RNAi because they can be easily titrated to control transgene copy number and are stably maintained as genomic integrants during subsequent cell replication (2, 12, 13). Therefore, we designed a single lentiviral vector to deliver Cas9, a sgRNA, and a puromycin selection marker into target cells (lentiCRISPR) (Fig. 1A). The ability to simultaneously deliver Cas9 and sgRNA through

a single vector enables application to any cell type of interest, without the need to first generate cell lines that express Cas9.

To determine the efficacy of gene knockout by lentiCRISPR transduction, we tested six sgRNAs targeting enhanced green fluorescent protein (EGFP) in a human embryonic kidney (HEK) 293T cell line containing a single copy of EGFP (fig. S1). After transduction at a low multiplicity of infection (MOI = 0.3) followed by selection with puromycin, lentiCRISPRs abolished EGFP fluorescence in $93 \pm 8\%$ (mean $\pm$ SD) of cells after 11 days (Fig. 1B). Deep sequencing of the EGFP locus revealed a $92 \pm 9\%$ indel frequency ($n \geq 10^4$ sequencing reads per condition) (fig. S2). In contrast, transduction of cells with lentiviral vectors expressing EGFP-targeting shRNA led to incomplete knockdown of EGFP fluorescence (Fig. 1C).

Given the high efficacy of gene knockout by lentiCRISPR, we tested the feasibility of conducting genome-scale CRISPR-Cas9 knockout (GeCKO) screening with a pooled lentiCRISPR library. We designed a library of sgRNAs targeting 5' constitutive exons (Fig. 2A) of 18,080 genes in the human genome with an average coverage of 3 to 4 sgRNAs per gene (table S1), and each target site was selected to minimize off-target modification (14) (see supplementary text).

To test the efficacy of the full GeCKO library at achieving knockout of endogenous gene targets, we conducted a negative selection screen by profiling the depletion of sgRNAs targeting essential survival genes (Fig. 2A). We transduced the human melanoma cell line A375 and the human



embryonic stem cells, respectively. Shift in the 14-day curve represents the depletion in a subset of sgRNAs. (D and E) The five most significantly depleted gene sets in A375 cells [nominal $P < 10^{-5}$, false discovery rate (FDR)-corrected $q < 10^{-5}$] and HUES62 cells (nominal $P < 10^{-5}$, FDR-corrected $q < 10^{-3}$) identified by GSEA (15).

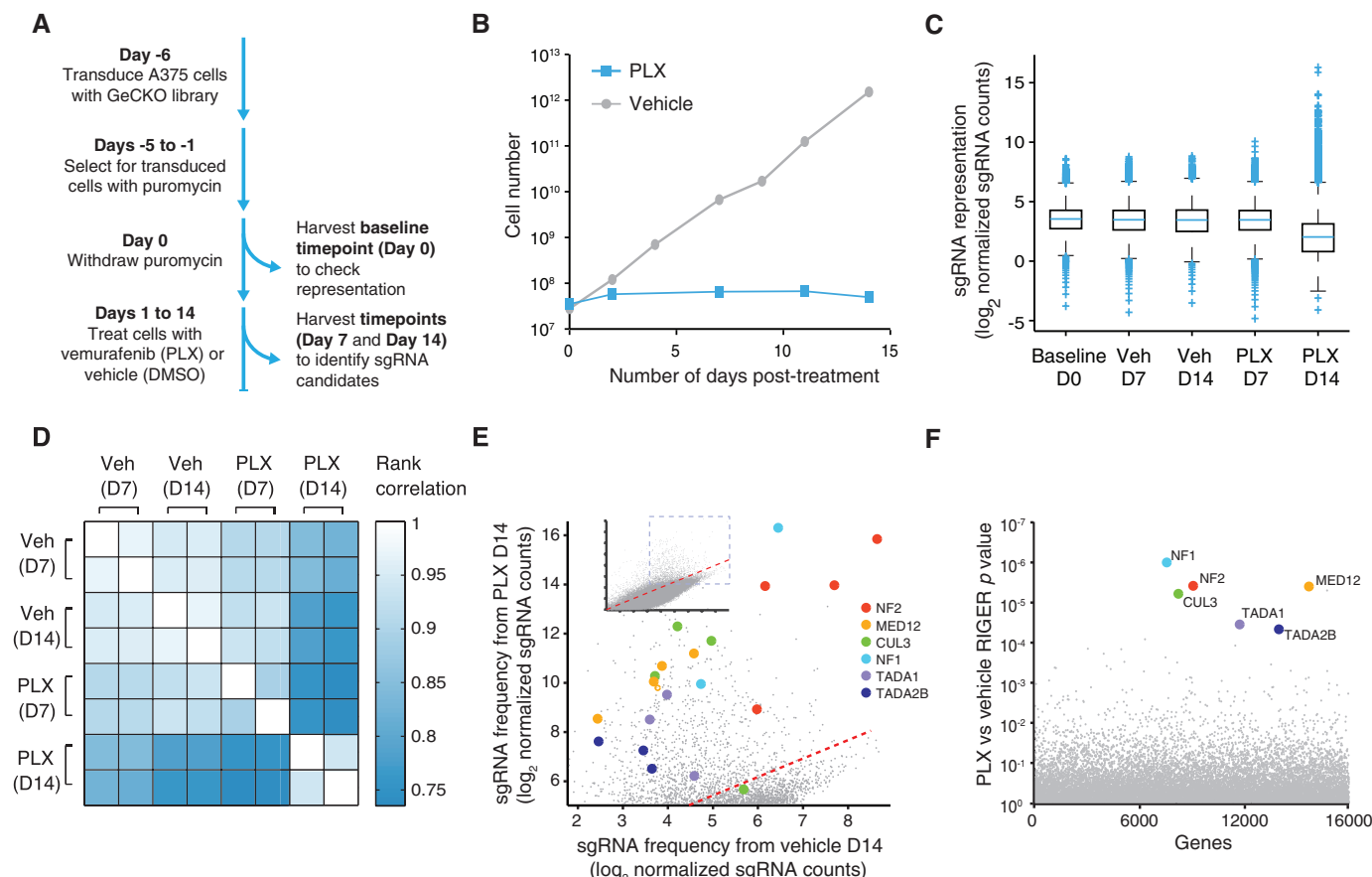


Fig. 3. GeCKO screen in A375 melanoma cells reveals genes whose loss confers PLX resistance. (A) Timeline of PLX resistance screen in A375 melanoma cells. (B) Growth of A375 cells when treated with dimethyl sulfoxide (DMSO) or PLX over 14 days. (C) Box plot showing the distribution of sgRNA frequencies at different time points, with and without PLX treatment (vehicle is DMSO). The box extends from the first to the third quartile with the whiskers denoting 1.5 times the interquartile range. Enrichment of

specific sgRNAs: 7 days of PLX treatment, 1 sgRNA greater than 10-fold enrichment; 14 days of PLX treatment, 379 and 49 sgRNAs greater than 10-fold and 100-fold enrichment, respectively. (D) Rank correlation of normalized sgRNA read count between biological replicates and treatment conditions. (E) Scatterplot showing enrichment of specific sgRNAs after PLX treatment. (F) Identification of top candidate genes using the RIGER P value analysis.

stem cell line HUES62 with the GeCKO library at a MOI of 0.3. As expected, deep sequencing (figs. S3 and S4) 14 days after transduction revealed a significant reduction in the diversity of sgRNAs in the surviving A375 and HUES62 cells (Fig. 2, B and C) (Wilcoxon rank sum test, $P < 10^{-10}$ for both cell types). Gene set enrichment analysis (GSEA) (15) indicated that most of the depleted sgRNAs targeted essential genes such as ribosomal structural constituents (Fig. 2, D and E, and tables S2 and S3). The overlap in highly depleted genes and functional gene categories between the two cell lines (fig. S5) indicates that GeCKO can identify essential genes and that enrichment analysis of depleted sgRNAs can pinpoint key functional gene categories in negative selection screens.

To test the efficacy of GeCKO for positive selection, we sought to identify gene knockouts that result in resistance to the BRAF protein kinase inhibitor vemurafenib (PLX) in melanoma (16) (Fig. 3A). Exposure to PLX resulted in growth arrest of transduced A375 cells, which harbor the V600E gain-of-function BRAF mutation (17) (Fig. 3B), therefore enabling the enrichment of a

small group of cells that were rendered drug-resistant by Cas9:sgRNA-mediated modification. After 14 days of PLX treatment, the sgRNA distribution was significantly different when compared with vehicle-treated cells (Fig. 3C) (Wilcoxon rank-sum test, $P < 10^{-10}$) and clustered separately from all other conditions (Fig. 3D and fig. S6).

For a subset of genes, we found enrichment of multiple sgRNAs that target each gene after 14 days of PLX treatment (Fig. 3E), suggesting that loss of these particular genes contributes to PLX resistance. We used the RNAi Gene Enrichment Ranking (RIGER) algorithm to rank screening hits by the consistent enrichment among multiple sgRNAs targeting the same gene (Fig. 3F and table S4) (12). Our highest-ranking genes included previously reported candidates *NF1* and *MED12* (18, 19) and also several genes not previously implicated in PLX resistance, including neurofibromin 2 (*NF2*), Cullin 3 E3 ligase (*CUL3*), and members of the STAGA histone acetyltransferase complex (*TADA1* and *TADA2B*). These candidates yield new testable hypotheses regarding PLX resistance mechanisms (see supplementary

text). For example, *NF1* and *NF2*, although unrelated in sequence, are each mutated in similar but distinct forms of neurofibromatosis (20). In addition, epigenetic dysregulation resulting from mutations in the mechanistically related STAGA and Mediator complexes (21) may have a role in acquired drug resistance. All of these hits were also identified through a second independent library transduction (figs. S7 and S8 and tables S5 and S6).

A similar screen to identify PLX drug resistance in A375 cells was previously conducted using a pooled library of 90,000 shRNAs (19). To compare the efficacy and reliability of genome-scale shRNA screening with GeCKO, we used several methods to evaluate the degree of consistency among the sgRNAs or shRNAs targeting the top candidate genes. First, we plotted the P values for the top 100 hits using either RIGER (Fig. 4A) or redundant siRNA activity (RSA) (fig. S9) scoring. Lower P values for the GeCKO versus shRNA screen indicate better scoring consistency among sgRNAs. Second, for the top 10 RIGER hit genes, $78 \pm 27\%$ of sgRNAs targeting each gene ranked among the top 5% of enriched sgRNAs, whereas

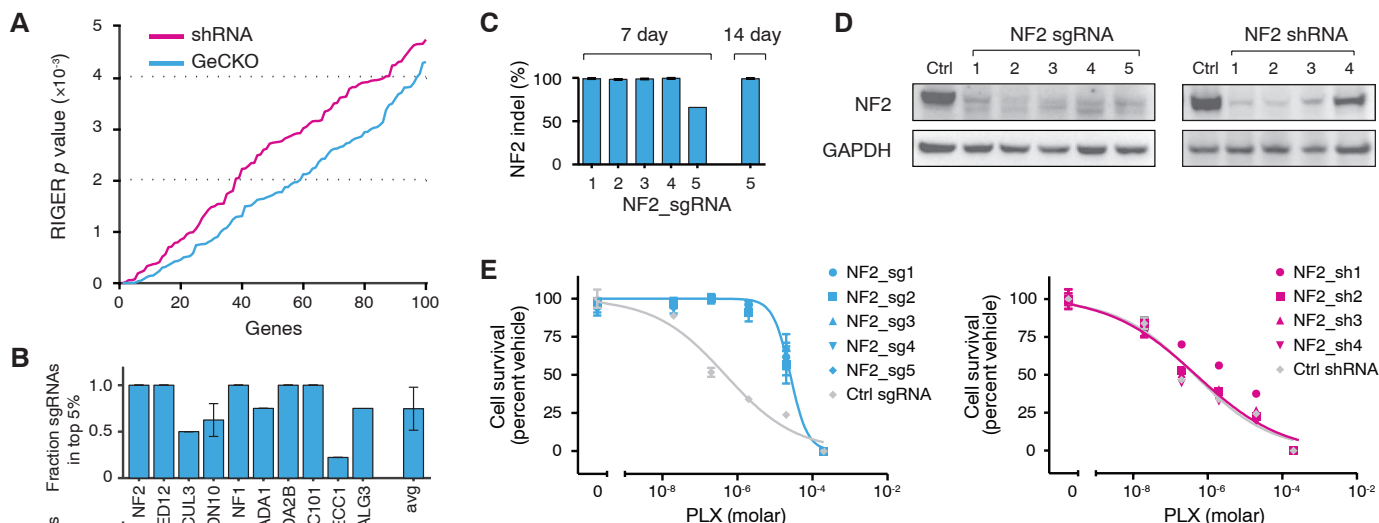


Fig. 4. Comparison of GeCKO and shRNA screens and validation of neurofibromin 2 (NF2).

(A) RIGER P values for the top 100 hits from GeCKO and shRNA (19) screens for genes whose loss results in PLX resistance. Analysis using the RSA algorithm shows a similar trend (fig. S9). (B) For the top 10 RIGER hits, the percent of unique sgRNAs (top) or shRNAs (bottom) targeting each gene that are in the top 5% of all enriched sgRNAs or shRNAs. (C) Deep-sequencing analysis of lentiCRISPR-mediated indel at the *NF2* locus. (D) A375 cells transduced with *NF2*-targeting lentiCRISPR and shRNA vectors both show a decrease in *NF2* protein levels. (E) Dose-response curves for A375 cells transduced with individual *NF2*-targeting lentiCRISPR or shRNA vectors. Controls were EGFP-targeting lentiCRISPR or null-hairpin shRNA vectors. Cells transduced with *NF2*-targeting lentiCRISPRs show a significant increase ($F_{1,8} = 30.3$, $P < 0.001$, $n = 4$ replicates) in the half-maximal effective concentration (EC_{50}), whereas cells transduced with *NF2*-targeting shRNA vectors do not ($F_{1,8} = 0.47$, $P = 0.51$, $n = 4$ replicates).

20 $\pm$ 12% of shRNAs targeting each gene ranked among the top 5% of enriched shRNAs (Fig. 4B).

We validated top-ranking genes from the GeCKO screen individually using 3 to 5 sgRNAs (Fig. 4, C to E, and figs. S10 and S11). For *NF2*, we found that 4 out of 5 sgRNAs resulted in >98% allele modification 7 days after transduction, and all 5 sgRNAs showed >99% allele modification 14 days after transduction (Fig. 4C). We compared sgRNA and shRNA-mediated protein depletion and PLX resistance using Western blot (Fig. 4D) and cell growth assays (Fig. 4E). Interestingly, although all five sgRNAs conferred resistance to PLX, only the best shRNA achieved sufficient knockdown to increase PLX resistance (Fig. 4E), suggesting that even low levels of *NF2* are sufficient to retain sensitivity to PLX. Additionally, sgRNAs targeting *NF1*, *MED12*, *CUL3*, *TADA1*, and *TADA2B* led to a decrease in protein expression and increased resistance to PLX (figs. S10 and S11). Deep sequencing confirmed a high rate of mutagenesis at targeted loci (figs. S12 and S13), with a small subset of off-target sites exhibiting indels (figs. S14 to S16), which may be alleviated using an offset nicking approach (22, 23) that was recently shown to reduce off-target modifications (22).

GeCKO screening provides a mechanistically distinct method from RNAi for systematic perturbation of gene function. Whereas RNAi reduces protein expression by targeting RNA, GeCKO introduces loss-of-function mutations into genomic DNA. Although some indel mutations are expected to maintain the reading frame, homozygous knockout yields high screening sensitiv-

ity, which is especially important in cases where incomplete knockout retains gene function. In addition, RNAi is limited to transcripts, whereas Cas9:sgRNAs can target elements across the entire genome, including promoters, enhancers, introns, and intergenic regions. Furthermore, catalytically inactive mutants of Cas9 can be tethered to different functional domains (23–27) to broaden the repertoire of perturbation modalities, including genome-scale gain-of-function screening using Cas9 activators and epigenetic modifiers. In the GeCKO screens presented here, the efficiency of complete knockout, the consistency of distinct sgRNAs, and the high validation rate for top screen hits demonstrate the potential of Cas9:sgRNA-based technology to transform functional genomics.

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Supplementary Materials

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Break-Induced Replication Repair of Damaged Forks Induces Genomic Duplications in Human Cells

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In budding yeast, one-ended DNA double-strand breaks (DSBs) and damaged replication forks are repaired by break-induced replication (BIR), a homologous recombination pathway that requires the Pol32 subunit of DNA polymerase delta. DNA replication stress is prevalent in cancer, but BIR has not been characterized in mammals. In a cyclin E overexpression model of DNA replication stress, *POLD3*, the human ortholog of *POL32*, was required for cell cycle progression and processive DNA synthesis. Segmental genomic duplications induced by cyclin E overexpression were also dependent on *POLD3*, as were BIR-mediated recombination events captured with a specialized DSB repair assay. We propose that BIR repairs damaged replication forks in mammals, accounting for the high frequency of genomic duplications in human cancers.

Activated oncogenes induce collapse and/or breakage of DNA replication forks (damaged forks), leading to DNA replication stress and DNA double-strand breaks (DSBs) (1, 2). To identify repair pathways for damaged forks, we performed a small interfering RNA (siRNA) screen monitoring DNA synthesis in a U2OS cell system, in which tetracycline withdrawal induces cyclin E overexpression and, subsequently, DNA replication stress (3, 4). The siRNA library targeted 690 genes implicated in DNA metabolism, and the cells were cultured with or without tetracycline and also with or without hydroxyurea (HU) to compare the responses to damaged versus stalled forks (5).

The screened genes were distributed into four clusters (fig. S1 and table S1). The first cluster—comprising genes important for DNA synthesis, specifically in cells overexpressing cyclin E—included *POLD3*, which encodes a subunit of DNA polymerase delta (6), and homologous recombination genes (7). The second cluster mediated the response to HU and included the *BLM* helicase and the *ATR* and *CHK1* checkpoint genes (8, 9). The third cluster included the genes whose depletion preferentially affected the cells that were exposed to HU while overexpressing cyclin E. However, no gene in this cluster had a strong phenotype. Finally, the last cluster encompassed the genes whose depletion did not affect the response to DNA replication stress.

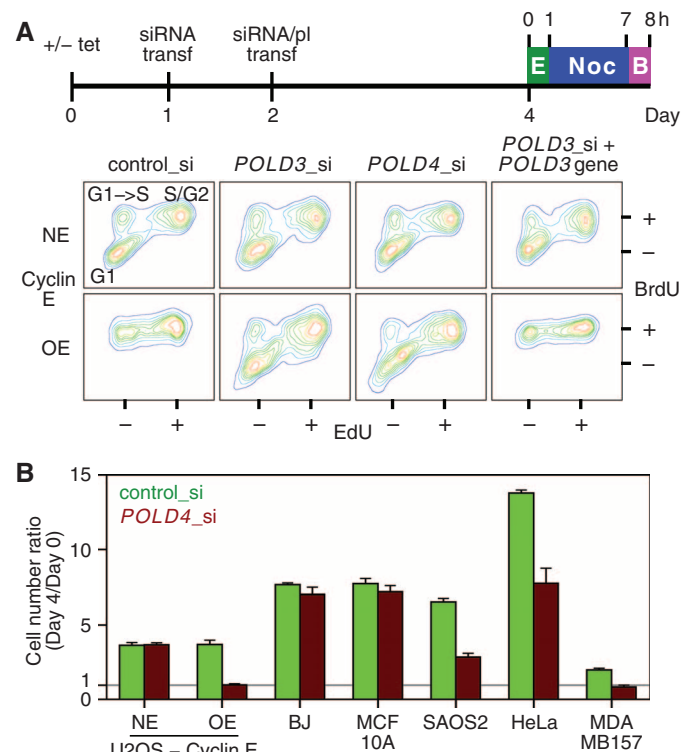
From the identified hits, we pursued *POLD3*, whose budding yeast ortholog, *POL32*, is essential for break-induced replication (BIR) (10–12) (fig. S2). We also examined *POLD4*, which encodes the fourth subunit of DNA polymerase delta (6), because in fission yeast the orthologs of *POLD3* and *POLD4* are both nonessential (13). Cells expressing normal or high levels of cyclin E were transfected with siRNA and, 3 days later, exposed to two thymidine-analog pulses (EdU and BrdU, respectively; 1 hour each, separated by 6 hours) to monitor cell cycle progression (Fig. 1A and fig. S3). As reported (14), cyclin E

overexpression enhanced the fraction of G₁ cells entering S phase during the 8-hour period (Fig. 1A and fig. S4). Depletion of *POLD3* or *POLD4* inhibited S phase entry in the cells overexpressing cyclin E but had no effect in cells expressing normal cyclin E levels (Fig. 1A and fig. S5). In a similar assay, depletion of *POLD3* or *POLD4* had no effect on S phase entry of cells treated with HU or aphidicolin (fig. S6). Because short-term exposure to HU or aphidicolin induces fork stalling, but not fork damage (15), we conclude that the functions of *POLD3* and *POLD4* relate to damaged forks.

Depletion of *POLD3* or *POLD4* also inhibited growth of U2OS cells overexpressing cyclin E ($P < 0.001$), whereas growth of cells expressing normal cyclin E levels was unaffected (Fig. 1B and fig. S7). Growth of SAOS2 osteosarcoma, HeLa cervical carcinoma, and MDA-MB157 breast carcinoma cells, all of which have DNA replication stress, was also inhibited after *POLD4* depletion ($P < 0.001$ for all), whereas growth of nontransformed cells, such as BJ fibroblasts and MCF10A mammary epithelial cells, was unaffected (Fig. 1B).

Next, we analyzed replication forks by DNA combing. In U2OS cells expressing normal cyclin E levels, most of the forks were ongoing (~60%), irrespective of *POLD3* or *POLD4* depletion. In cells overexpressing cyclin E, the fraction of ongoing forks was still higher (~45%) than the fraction of terminated forks (~28%). However, when *POLD3* or *POLD4* was depleted, the ongoing forks became a minority (~17%) and the terminated forks the majority (~47%),

Fig. 1. *POLD3* and *POLD4* are required for cell cycle progression in the presence of oncogene-induced DNA replication stress. (A) (Top) Experimental outline. tet, tetracycline; transf, transfected; pl, plasmid; E, EdU; B, BrdU; Noc, nocodazole. (Bottom) Flow cytometry analysis. U2OS cells expressed normal levels of cyclin E (NE) or overexpressed cyclin E (OE). si, siRNA; EdU→BrdU⁺, cells that remained in G₁ or were blocked at the G₁/S interface (G₁); EdU→BrdU⁺, cells that were in G₁ at 0 to 1 hour, but entered S phase by 7 to 8 hours (G₁→S); EdU+/BrdU⁺, cells that were in S phase at 0 to 1 hour (S/G₂). (B) Effect of *POLD4* depletion on cell growth. The cells were seeded on day 0, transfected with siRNA on day 1, and counted on day 4. Means and SDs (error bars) from three independent experiments are shown.



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suggesting that *POLD3* and *POLD4* are important for fork processivity when cyclin E is overexpressed (Fig. 2A). As reported (16), cyclin E overexpression reduced replication fork speeds

(Fig. 2B and fig. S8). Depletion of *POLD3* or *POLD4* did not affect fork speeds in cells with normal cyclin E levels, but the forks traveling slower than 0.5 kb/min were preferentially tar-

geted in cells overexpressing cyclin E ($P < 0.005$) (Fig. 2B). Thus, slow forks may be different from fast forks.

In budding yeast, BIR repairs damaged replication forks and also one-ended DNA DSBs (11, 12). To explore the role of *POLD3* and *POLD4* in DSB repair, various human cell lines transfected with siRNA were exposed to ionizing radiation, and 53BP1 and replication protein A (RPA) foci, surrogate markers for unrepaired DNA DSBs and DNA replication stress, respectively (17), were scored. Both types of foci persisted longer in the cells, in which *POLD3* or *POLD4* was depleted (Fig. 3A and fig. S9) (U2OS cells: $P < 0.01$ for 53BP1 foci at 16 hours and for RPA foci at 24 hours).

The role of *POLD3* and *POLD4* in DNA DSB repair was further examined using green fluorescent protein (GFP)-based reporters, in which DSBs were induced by the nuclease I-SceI. The reporters monitoring synthesis-dependent strand annealing (SDSA) and single-strand annealing (SSA) were supplemented with a newly developed BIR reporter, in which the sequence homology was on only one side of the DSB, to prevent repair by SDSA, and the homologous GFP sequences were in opposite orientations, to prevent repair by SSA (Fig. 3B). Repair of the I-SceI-induced DSB by BIR would start with invasion of the broken end into an uncut

Fig. 2. *POLD3* and *POLD4* are required for fork processivity under conditions of oncogene-induced DNA replication stress.

(A) Distribution of replication forks. U2OS cells expressed normal levels of cyclin E (NE) or overexpressed cyclin E (OE). Replication forks were scored as ongoing (Ong), terminated (Term), or newly fired (NewF). The data represent two independent experiments for *POLD3* depletion and one experiment for *POLD4* depletion. (B) Distribution of replication speeds of ongoing DNA replication forks as a function of cyclin E expression levels and *POLD3/POLD4* depletion. The percentages are relative to the total number of forks counted (ongoing, terminated, and newly fired), but only data for the ongoing forks are presented. Error bars in (A) and (B) indicate SDs.

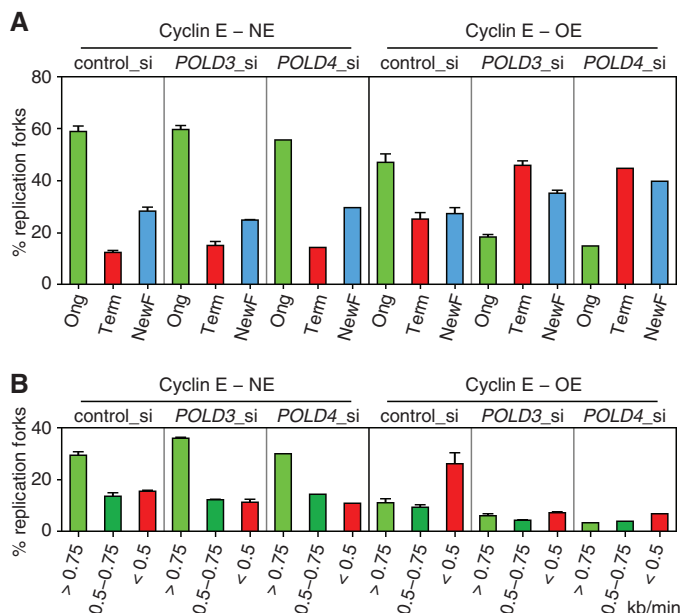
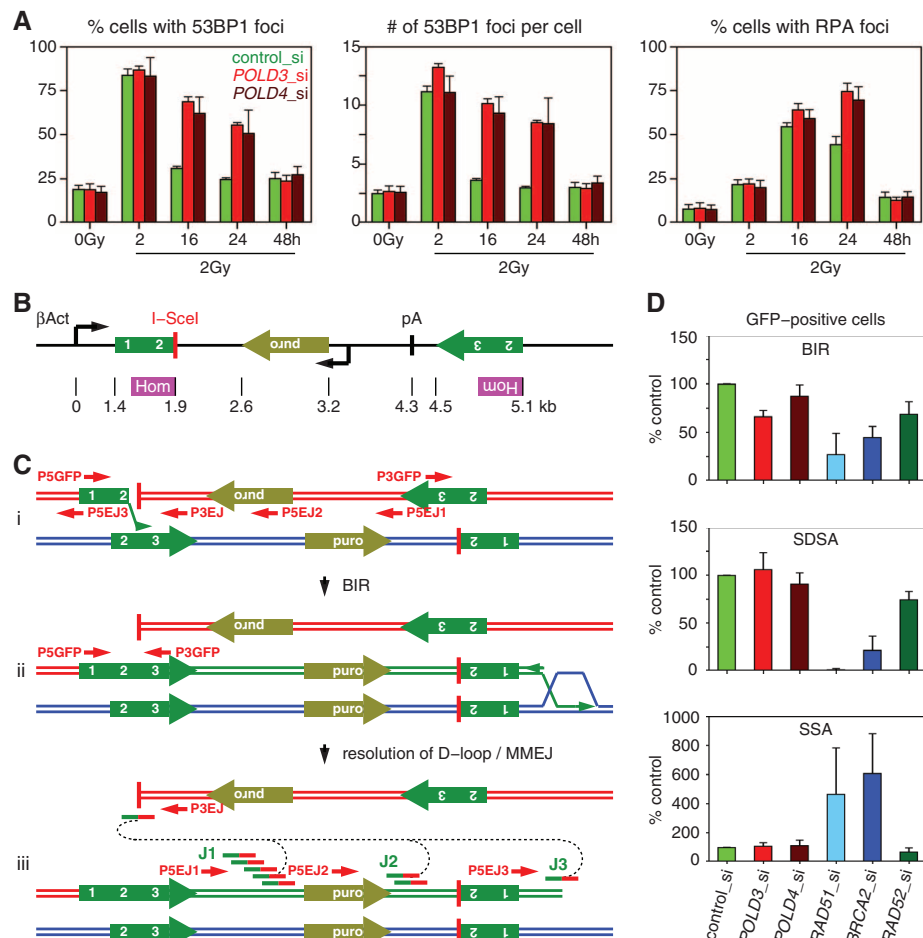


Fig. 3. Role of *POLD3* and *POLD4* in DNA DSB repair.

(A) Percentages of cells with 53BP1 or RPA foci and average number of 53BP1 foci per cell in nonirradiated [0 gray (Gy)] and irradiated (2 Gy; 2, 16, 24, and 48 hours after irradiation) U2OS cells. Error bars indicate SDs. (B) GFP-based reporter plasmid to monitor BIR. A β -actin (β Act) promoter drives expression of N-terminal GFP sequences. The C-terminal GFP sequences are in the opposite orientation. The area of homology (Hom, 400 bps) is limited to one side of the I-SceI-induced break. Position 0 kb refers to the β -actin promoter transcription start site. pA, poly A site. (C) Expected and observed products after repair by BIR. Three steps are shown: (i) homology search, (ii) strand extension, and (iii) dissolution of the D-loop and joining of the free DNA ends by MMEJ. P5GFP and P3GFP, primers to amplify GFP; P5EJ1, P5EJ2, P5EJ3, and P3EJ, primers to amplify MMEJ-generated junctions of types J1, J2, and J3, respectively. The short green and red lines indicate eight observed junctions. (D) Effect of *POLD3* or *POLD4* depletion on repair of DNA DSBs by BIR, SDSA, and SSA. Means and SDs (error bars) from experiments performed in quadruplicate are relative to control siRNA-transfected cells.



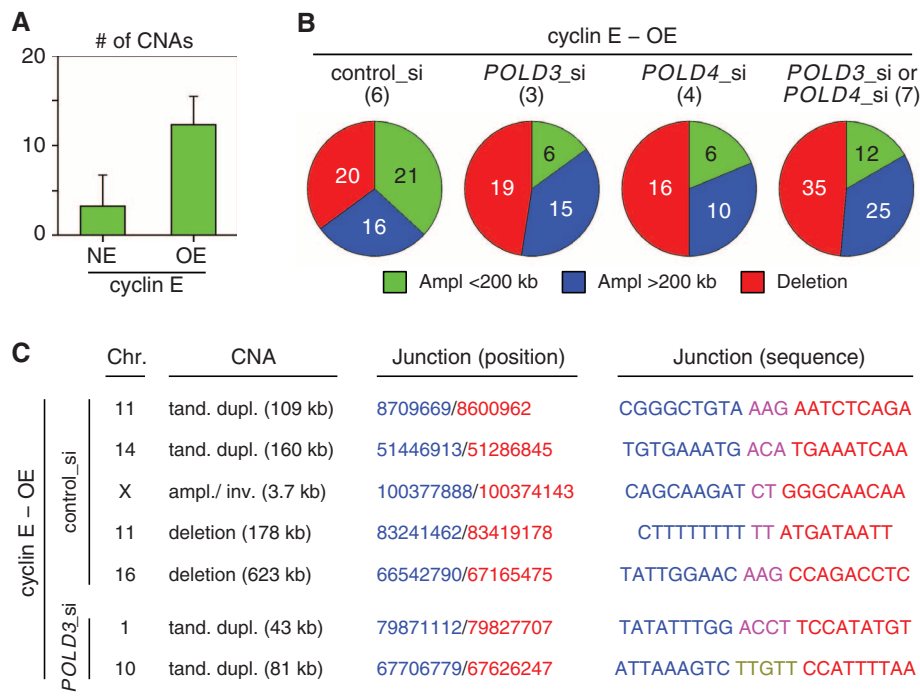


Fig. 4. Cyclin E overexpression-induced genomic rearrangements. (A) Number of CNAs induced over a 3-week period in U2OS cells expressing normal or high levels of cyclin E (NE and OE, respectively). Means and SDs (error bars) are from three clones per group. (B) Number of different types of CNAs (amplifications smaller than 200 kb, amplifications greater than 200 kb, and deletions) induced in U2OS cells overexpressing cyclin E over a 3-week period during which the cells were transfected every 3 days with siRNA. The number of cell clones analyzed is in parentheses. The data from the *POLD3* and *POLD4*-depleted clones are also shown grouped together. (C) Sequences of breakpoint junctions in two cyclin E-overexpressing clones, one of which had been transfected with control siRNA and one with siRNA targeting *POLD3*. The type of CNA associated with these junctions, its size (in kb), and the affected chromosome (Chr.) are indicated. The two joined sequences are colored blue and red, respectively; the microhomologies or small insertions at the junction are colored purple and olive green, respectively. tand. dupl., tandem duplication; ampl./inv., amplification/inversion.

homologous template (Fig. 3C, i). Conservative replication initiated from the invading strand (19) would restore the *GFP* coding sequence (Fig. 3C, ii). Then, the low processivity of DNA polymerase delta, which is the polymerase at the invading strand (20), would lead to replication fork disengagement, and the newly created DNA end would be joined to the only other available free end, the one generated by I-SceI (Fig. 3C, iii).

The BIR reporter described above was stably integrated in U2OS cells, and DSBs were induced by expressing I-SceI. Sequencing of polymerase chain reaction products prepared using primers specific for *GFP* (primers P5GFP and P3GFP, respectively) (Fig. 3C) showed accurate recombination. Next, the predicted end-joining-generated junctions were amplified using forward primers downstream of *GFP* (primers P5EJ1, P5EJ2 or P5EJ3) and a reverse primer downstream of the I-SceI cleavage site (primer P3EJ) (Fig. 3C). The sequences of eight breakpoint junctions were consistent with stochastic dissociation of the BIR-initiated fork from the template and with variable resection at the I-SceI-induced break (Fig. 3C, iii). Microhomologies [2 to 4 base pairs (bps)] or small insertions were present in all breakpoint

junctions (fig. S10), suggesting repair of the free ends by microhomology-mediated end joining [(MMEJ), also known as backup-EJ or Alt-EJ], a Ku-independent break-repair mechanism (21). Thus, human cells can repair one-ended DNA DSBs by BIR.

Depletion of *POLD3* suppressed DSB repair by BIR ($P < 0.002$) but did not affect repair by SDSA or SSA (Fig. 3D), consistent with *POLD3* playing a role in processive synthesis at the invading DNA strand, as shown for yeast and *Drosophila* *POL32* (20, 22). In contrast, depletion of *POLD4* had no effect in any of the repair assays (Fig. 3D). Both *POLD3* and *POLD4* contain proliferating cell nuclear antigen-binding motifs, and both enhance DNA polymerase delta processivity in vitro (6, 23), but perhaps *POLD4* is less active than *POLD3*. In our assay, DNA synthesis of as little as 1 kb conferred GFP expression (Fig. 3C), but in *Drosophila* the effects of *POL32* on extension of the invading strand are barely evident, when synthesis is limited to 1 kb (22).

In budding yeast, *POL32* is required for induction of tandem duplications under conditions of DNA replication stress, and these duplications were attributed to BIR or to microhomology-

induced replication, a BIR-related mechanism (24). In fission yeast, duplications associated with fold-back inversions have also been attributed to BIR (25). By array-based comparative genomic hybridization (CGH), overexpression of cyclin E in U2OS cells for 3 weeks induced copy-number alterations (CNAs) (Fig. 4A) ($P < 0.05$, number of CNAs for cyclin E overexpression versus normal expression). To examine the effect of depleting *POLD3* or *POLD4* on the spectrum of CNAs, we repeated the experiment, except that during the period of cyclin E overexpression, the cells were transfected every 3 days with siRNA. In single-cell clones isolated from cells transfected with control siRNA, amplifications (presumably, duplications) less than 200 kb in length accounted for a third of all CNAs (Fig. 4B and table S2). However, in clones isolated from *POLD3*- or *POLD4*-depleted cells, the frequency of such amplifications decreased by half (Fig. 4B) ($P < 0.02$, control versus *POLD3* siRNA; $P < 0.08$, control versus *POLD4* siRNA; $P < 0.01$, control versus *POLD3* siRNA and *POLD4* siRNA grouped together). In yeast, BIR can lead to DNA synthesis of ~100 kb (26). Thus, duplications of up to 200 kb in human cells may represent BIR events, whereas the larger amplifications and deletions may arise from other repair mechanisms, such as non-allelic homologous recombination.

Polymerase chain reaction primers designed using the CGH array data failed to amplify CNA breakpoint junctions. Thus, genomic DNA from two clones was subjected to high-throughput paired-end sequencing. In the first clone, derived from cells transfected with control siRNA, five junctions were identified (Fig. 4C and figs. S11 and S12), revealing two head-to-tail tandem duplications, one duplication associated with a fold-back inversion, and two simple deletions. Microhomologies were present in all breakpoint junctions. In the second clone, derived from cells transfected with *POLD3* siRNA, two junctions were identified, revealing two head-to-tail tandem duplications, of which one had a microhomology junction and the other a 5-bp insertion (Fig. 4C and fig. S12).

The most frequent type of CNA in breast and ovarian cancers, representing one third of all somatic rearrangements, is tandem head-to-tail duplications with microhomology junctions (27, 28). Fold-back inversions are very common in pancreatic cancer (29). Both types of CNAs were observed in U2OS cells overexpressing cyclin E, and their frequency decreased after depleting *POLD3* or *POLD4*. Thus, BIR repair of damaged replication forks might explain the presence of segmental genomic duplications in human cancers (fig. S13). Notably, the *POLD3* gene is frequently amplified in human cancers (30), and its protein product is overexpressed in cancer cell lines (fig. S14).

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Supplementary Materials

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A Neural Mechanism Underlying Mating Preferences for Familiar Individuals in Medaka Fish

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Social familiarity affects mating preference among various vertebrates. Here, we show that visual contact of a potential mating partner before mating (visual familiarization) enhances female preference for the familiarized male, but not for an unfamiliarized male, in medaka fish. Terminal-nerve gonadotropin-releasing hormone 3 (TN-GnRH3) neurons, an extrahypothalamic neuromodulatory system, function as a gate for activating mating preferences based on familiarity. Basal levels of TN-GnRH3 neuronal activity suppress female receptivity for any male (default mode). Visual familiarization facilitates TN-GnRH3 neuron activity (preference mode), which correlates with female preference for the familiarized male. GnRH3 peptides, which are synthesized specifically in TN-GnRH3 neurons, are required for the mode-switching via self-facilitation. Our study demonstrates the central neural mechanisms underlying the regulation of medaka female mating preference based on visual social familiarity.

Social familiarization—the process of becoming familiar with an individual through visual or odor contact—affects mate pref-

erence in various species (1, 2). For example, female mating preference for novel males over familiar males has been demonstrated in guppies (1–3), whereas mating preference for familiar males has been reported in some rodents, such as prairie voles (4). The neural and molecular bases underlying how social familiarity affects mate preference, however, remain obscure. Here, we studied the effects of social familiarity on female mating preference and the underlying neural mechanisms in medaka fish (*Oryzias latipes*), a model animal used primarily in the field of molecular genetics (5).

Mating behavior in medaka comprises several sequential steps, including male courtship display and synchronized mating (6, 7) (fig. S1). If a female medaka is not receptive to male courtship, the female rejects the male by characteristic re-

sistance behavior, and the rejected male begins to display courtship behavior again (7). We assessed the degree of female receptivity for a male of interest by measuring the interval between the first male courtship behavior and the first mating (latency to mate). Shorter values of latency to mate correlate with higher female receptivity. We compared the latency to mate of two groups: Group 1 with visual familiarization (a pair of fish can see each other through a transparent wall) before being placed in the same tank for mating, and group 2 without visual familiarization (Fig. 1A and fig. S2b). Visual familiarization significantly decreased the latency to mate (group 1), indicating that visual familiarization enhances female receptivity (Fig. 1B). By contrast, familiarity had no effect on male mating activity (fig. S3). We then examined female preference for familiar males. In group 3, a female was familiarized with a male and then mated with an unfamiliar male instead of the familiarized male (fig. S2b). In group 4, mating partners were swapped between two pairs that had undergone visual familiarization (fig. S2b). The latencies to mate in groups 3 and 4 were similar to that in group 2 (Fig. 1B), suggesting that the female could identify her mating partner. In group 5, a female was mated with an unfamiliar male that had been familiarized with another female. The lack of enhancement in group 5 (Fig. 1B and fig. S2b) indicated that female receptivity was not enhanced for an unfamiliar male that had been visually familiarized with another female. Experiments with females in which both eyes had been removed showed that vision was required for female preference for a familiar male (Fig. 1C).

During our search for possible genes involved in female mating preference in medaka, we found two mutants (*cxc7* and *cxc4*) with defective mating behaviors in both sexes (Fig. 1, D and E, and fig. S4). Both mutants exhibited normal mobility, visual social interaction (mirror-approaching behavior), and schooling behaviors (figs. S5 to

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S7). By contrast, both mutants, as well as the *cxc7* morphant, exhibited constitutively enhanced female receptivity even when placed in group 2 (Fig. 1, D and E), indicating that these mutants did not exhibit female mating preference. This enhancement was detected regardless of whether the mating partner was a wild-type or a mutant male (Fig. 1E). *cxc7* and *cxc4* encode C-X-C motif chemokine receptors that bind to a common ligand, stroma cell-derived factor 1 (SDF1, also known as CXCL12) (8–10). Although CXCR7 and CXCR4 were originally identified as essential factors for primordial germ cell migration during embryogenesis (8), the above-described behavioral abnormalities were not due to defective primordial germ cell migration (figs. S8 to S10). We next analyzed neural migration in the *cxc* mutants, with particular focus on the gonadotropin-releasing hormone (GnRH) neurons, because *cxc4* mutations in zebrafish lead to the defective migration of GnRH neurons during embryogenesis (10). Visualization of three types of GnRH neurons (11) (table S1)—preoptic area (POA)-, midbrain-, and terminal-nerve (TN)-GnRH neurons (12)—in medaka embryos revealed that mutation of either *cxc7* or *cxc4* led to abnormal development (incomplete migration and ectopic axonal projection) of TN-GnRH3 neurons, but not of the two other types (Fig. 2 and fig. S11, b and c). Furthermore, *cxc7* (table S2) and *cxc4* mutants can produce progeny by mating with wild males and therefore exhibit normal fertility, indicating the presence of intact POA-GnRH1 neurons. To examine the possible involvement of TN-GnRH3 neurons in female preference, we performed laser ablation of TN-GnRH3 neurons during the embryonic stage (Fig. 3A and fig. S11a) and tested the mating behavior of the ablated fish in the adult stage. The TN-GnRH3 neuron-ablated females exhibited constitutively enhanced female receptivity (Fig. 3B), similar to the behavioral phenotype of the *cxc* mutants, indicating that TN-GnRH3 neurons, by default, suppress female receptivity, and ablation of the TN-GnRH3 neurons releases this suppression.

To investigate how GnRH3 peptides synthesized in the TN-GnRH3 neurons mediate the regulation of female receptivity to unfamiliar males, we generated a *gnrh3* mutant (*gnrh3*^{S27P/S27P}) in which a conserved serine residue at amino acid position 27 was changed to proline (fig. S12). This mutant peptide exhibited reduced ligand activity for all GnRH receptor subtypes (1, 2, and 3), as assessed by luciferase assay in cultured cells (Fig. 3C). *gnrh3*^{S27P/S27P} females exhibited constitutively suppressed female receptivity in both groups 1 and 2 (Fig. 3D), in contrast to *cxc* mutants (Fig. 1E) and TN-GnRH3 neuron ablated females (Fig. 3B). This finding indicates that GnRH3 peptides are required for the female preference for familiar males, but not for the suppression of female receptivity. Thus, GnRH3 peptides in TN-GnRH3 neurons are required for switching from the default mode (suppression of female receptivity) to preference mode (female preference for familiar males). The *gnrh3*^{S27P/S27P} females

exhibited normal mirror-approaching behavior, schooling behaviors, and reproductive cycle (fig. S13 and table S2).

To investigate the physiological basis of “mode-switching” of the TN-GnRH3 neurons, we examined the correlation between TN-GnRH3 neural activity and female mating preference for familiar males. TN-GnRH3 neurons have spontaneous regular pacemaker activity (12, 13). Whether the pacemaker activity of TN-GnRH3 neurons is related to the regulation of sexual behavior, however, is unclear. Loose-patch recordings revealed

that visual familiarization significantly facilitated female TN-GnRH3 neuron activity (Fig. 3, E and F, and fig. S14a). Pacemaker frequencies were low, irrespective of familiarization, in sexually premature and old postmature females, both of which are incapable of reproducing (Fig. 3F). Pacemaker frequencies positively correlated with the degree of female receptivity, suggesting that facilitation of the pacemaker activity enhanced female preference for familiar males. Pacemaker frequencies were also low, irrespective of familiarization, in *gnrh3*^{S27P/S27P} (Fig. 3, E and F, and

Fig. 1. Regulation of female preference in wild-type and mutant medaka. (A) Experimental procedure for groups 1 and 2. Group 1: The female saw the male before mating. Group 2: The female did not see the male. (B) Enhanced female preference for the familiar male (fig. S2b). G3: Female familiarized with a male other than its mating partner. G4: Swap between two pairs with visual familiarization. G5: Male familiarized with a female other than the mating partner. Latency to mate: interval between the first courtship and mating (fig. S2d). WT(d-r): wild-type d-r strain. Mean ± SEM [one-way analysis of variance (ANOVA); Scheffé’s post hoc comparison, ***P* < 0.01, **P* < 0.05]. (C) Constitutively suppressed female receptivity in females in which both eyes had been removed. Sham fish were injured just above the eyes. Single and double: One or two eyes had been removed, respectively. (D and E) Constitutively enhanced female receptivity in *cxc7* morphants (D) and *cxc* mutants (E). WT: wild-type Cab strain. *cxc4* (XX): Sex-reversed XX males. (C to E) Mean ± SEM (Student’s *t* test: ***P* < 0.01, **P* < 0.05).

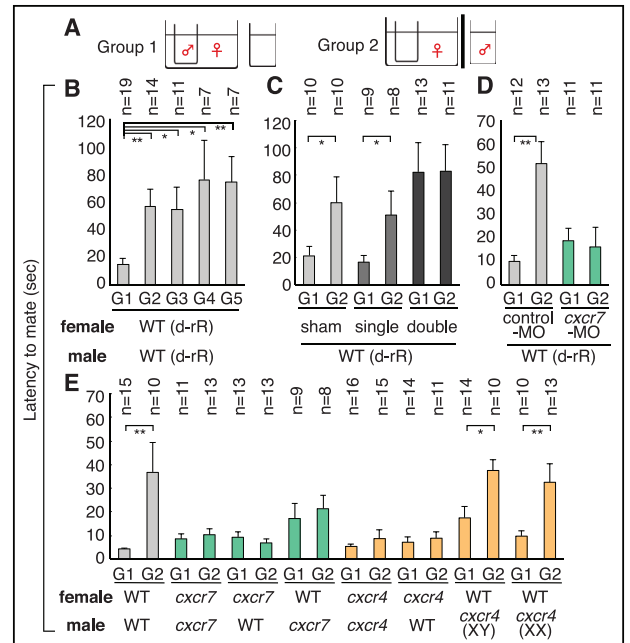


Fig. 2. GnRH neural development in *cxc* mutants. (A) Abnormal migration of the TN-GnRH3 neurons in *cxc* morphants in STIII, transparent medaka (26). White arrowheads indicate ectopic location of progenitor TN-GnRH3 neurons. Left: Embryos with GFP-labeled neurons visualized by Tg(*gnrh3:gfp*). Right: Magnified insets shown in the confocal photomicrographs (movies S2 and S3). (B) Normal migration of the POA-GnRH1 neurons visualized by Tg(*gnrh1:gfp*) in *cxc* morphants. Abnormal migration of the TN-GnRH3 neurons visualized by Tg(*gnrh3:gfp*) in *cxc7*-*l* is shown in fig. S11c. Scale bars, 100 μm. The diagrams below show the axonal projection pattern of TN-GnRH3 neurons (left) and POA-GnRH1 neurons (right) in the adult brain.

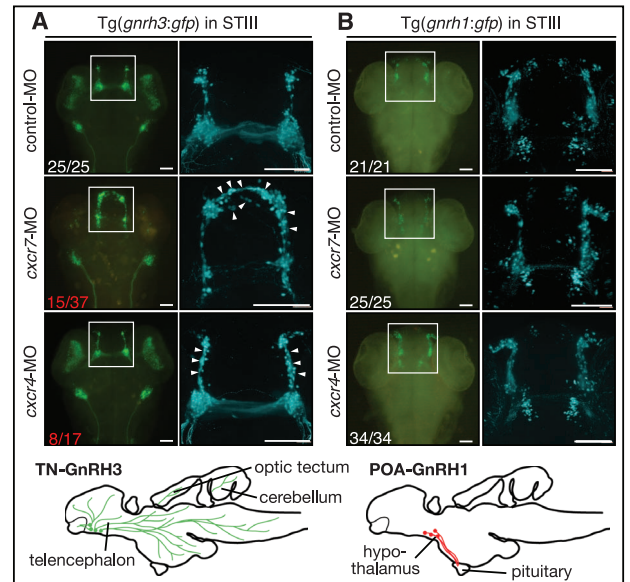


fig. S14a), indicating that GnRH3 peptides facilitate the pacemaker frequencies, which may be required for the female preference for familiar males.

Finally, we examined female preference for familiar males over unfamiliar males in medaka and the possible involvement of TN-GnRH3 neurons in mate preference. We performed a paternity test using three fish, one female and two males. Of the two males, one was wild-type and the other expressed green fluorescent protein (GFP) in the primordial germ cells, Tg(*olvas:gfp*), which allowed

for genotyping progeny on the basis of GFP detection and determination of the winner and loser between the two males (figs. S15 to S17). First, the two males were separated before mating under the set 1 conditions (Fig. 4A), in which both wild-type and Tg(*olvas:gfp*) males were visually familiarized with the female before mating. In set 1, the winning percentage of Tg males was ~60% (Fig. 4B). The three fish were then separated under different conditions (Fig. 4A) and placed together again for the mating assay. When the Tg, but not the wild-type, male was familiarized with

the female (set 2), the Tg winning percentage increased, and vice versa (set 3; Fig. 4B). Visual familiarization also affected male mating success when pairs of males differing in body size were used, i.e., one being stronger than the other (fig. S17, a and c). These findings indicate that a female chooses a familiar male as a mating partner (fig. S17b). *cxcr7*^{-/-} or *gnrh3*^{S27P/S27P} females did not exhibit a preference (Fig. 4B), strongly suggesting that the TN-GnRH3 neurons are essential for female preference in medaka (fig. S17, d and e).

On the basis of our findings, we propose a model for a bimodal TN-GnRH3 neuron functional mechanism in the central nervous system (Fig. 4C and fig. S18): TN-GnRH3 neurons, by default, have a basal level of pacemaker frequency (2 to 3 Hz) that suppresses female receptivity to any mates. Neurotransmitters or neuromodulators other than GnRH3 peptides, such as NPFF (fig. S19, a to c) (14) and glutamate (15), may mediate this suppression of female receptivity. GnRH3 peptides are critical for facilitation of TN-GnRH3 neurons depending on visual familiarization. Recent physiological studies demonstrated that GnRH3 peptides facilitate the pacemaker activity of TN-GnRH3 neurons by an autocrine or paracrine effect (12, 13). It is thus plausible that visual familiarization stimulates the release of GnRH3 peptides, which causes the self-facilitation of TN-GnRH3 neurons via a positive feedback mechanism. Visual familiarization that lasts for a long period of time (>6 hours) facilitates the pacemaker activity above a certain threshold (4 to 5 Hz; preference mode) and then activates mating preferences for the familiar male (fig. S18a). Considering that TN-GnRH3 neurons project to the visual system in medaka fish (fig. S19, d to i), it is reasonable to expect that GnRH3 peptides directly modulate the medaka visual system, which is involved in female preference. Previously, extensive electrophysiological and histological studies on songbirds (16) and frogs (17) revealed networks of neural pathways in the central brain regions underlying mate choice based on acoustic communication. This work, however, indicates involvement of the GnRH system in mate choice based on visual recognition.

Previous studies of female sexual behaviors have demonstrated that hormones such as GnRH peptides (18), sex steroids (19), and prostaglandin (20, 21) regulate female receptivity and/or mate preference in various vertebrates. To date, studies on GnRH systems have focused mainly on the hypothalamic-POA GnRH system, which mediates the hypothalamic-pituitary-gonadal axis. Little attention, however, has been given to the extrahypothalamic GnRH system (12) owing to the difficulty of genetic dissection (table S1), although extrahypothalamic GnRH neurons have also been anatomically identified in various vertebrate species, including mammals (22). As TN-GnRH3 neurons project their axons widely throughout the entire brain (fig. S19, d to i) (12), the extrahypothalamic TN-GnRH3 neurons are independent of the regulation of reproduction.

Fig. 3. Requirement for TN-GnRH3 neurons and GnRH3 peptides in female preference regulation.

(A) Laser ablation of the TN-GnRH3 neurons. White arrowheads indicate progenitor TN-GnRH3 neurons. Sham ablation: Regions adjacent to the TN regions were exposed to laser irradiation (fig. S11a) (27). GFP signals immediately after [left: 3 days postfertilization (dpf)] and 1 day after (right) laser irradiation were observed. (B) Constitutively enhanced female receptivity in the TN-GnRH3 neuron ablated female. (C) Decreased responses of the GnRH receptors (1, 2, and 3) for mutant GnRH3 peptides, which were measured by *SRE* promoter-driven luciferase assay. Mean $\pm$ SEM. Significant differences were detected between GnRH3 WT and S27P (two-way ANOVA: $P < 0.001$) in all receptors. (D) Constitutively suppressed female receptivity in *gnrh3*^{S27P/S27P}. (E) Representative electrophysiological recordings from TN-GnRH3 neurons in mature females and *gnrh3*^{S27P/S27P} mutants. (F) Regulation of pacemaker frequency dependent on visual familiarization. Low frequencies in premature (pre) females, old postmature (post) females, and *gnrh3*^{S27P/S27P} females. (B and D) Mean $\pm$ SEM (Student's *t* test: $*P < 0.05$). (F) One-way ANOVA: Scheffé's post hoc comparison, a and b, $P < 0.05$; a and c, $P < 0.01$; a and d, $P < 0.001$.

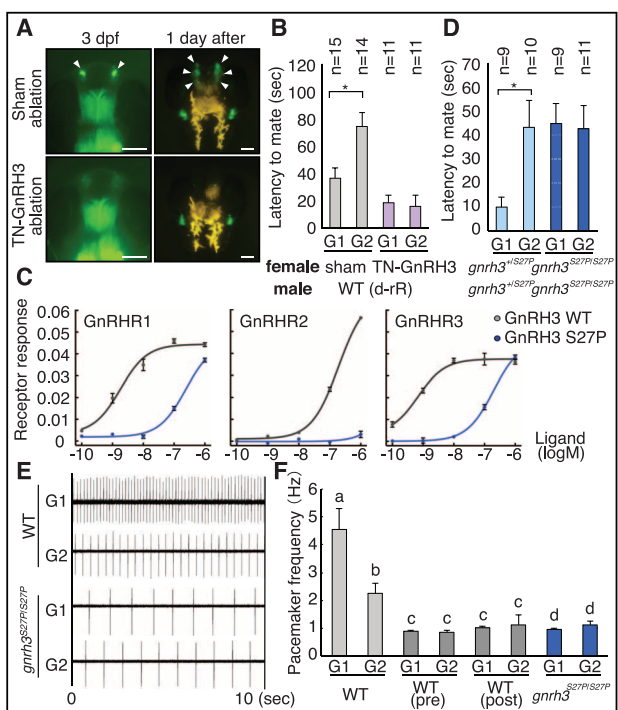
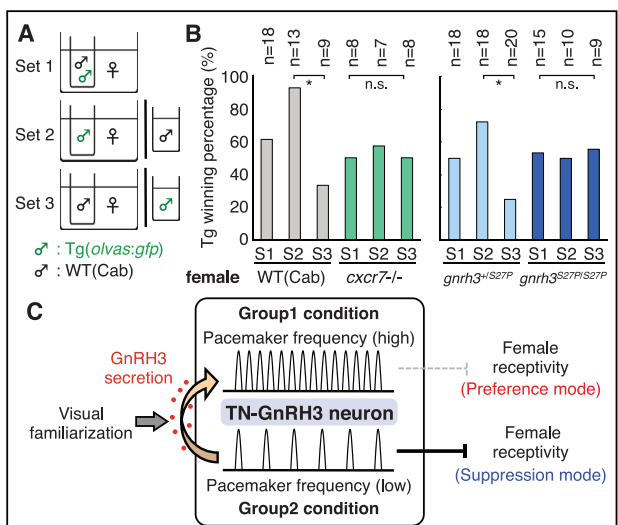


Fig. 4. Quantitative analyses of female preference.

(A) Separation conditions for female preference test. Set1: Female familiarized with both males. Set2: Female familiarized with the Tg (*olvas:gfp*) male. Set3: Female familiarized with the wild-type male. (B) Female preference for the familiar male and loss of female preference in the mutants. S1 to S3: set 1 to 3. Mean $\pm$ SEM (Steel-Dwass' test: $*P < 0.05$; n.s., not significant). (C) A model for a bimodal TN-GnRH3 neuron functional mechanism.



Further, our findings indicate that the role of TN-GnRH3 may be closely associated with female preference for a visually recognized individual. Individual recognition is central to many types of cooperative interactions (23), social decision-making in pair-bonding (4), kin recognition (1, 24), and social hierarchy (25). The present findings shed new light on the importance of TN-GnRH3 neurons for female preference, as well as for individual recognition, for social neuroscience.

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Supplementary Materials

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Materials and Methods

Figs. S1 to S19

Tables S1 and S2

Movies S1 to S3

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Pregnenolone Can Protect the Brain from Cannabis Intoxication

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Pregnenolone is considered the inactive precursor of all steroid hormones, and its potential functional effects have been largely uninvestigated. The administration of the main active principle of *Cannabis sativa* (marijuana), Δ^9 -tetrahydrocannabinol (THC), substantially increases the synthesis of pregnenolone in the brain via activation of the type-1 cannabinoid (CB₁) receptor. Pregnenolone then, acting as a signaling-specific inhibitor of the CB₁ receptor, reduces several effects of THC. This negative feedback mediated by pregnenolone reveals a previously unknown paracrine/autocrine loop protecting the brain from CB₁ receptor overactivation that could open an unforeseen approach for the treatment of cannabis intoxication and addiction.

Steroid hormones are important modulators of brain activity and behavior (1–4). Steroids play crucial roles in regulating physiological activities such as food intake, wakening, reproduc-

tion, and sexual behavior and participate in the regulation of mood and memory. Steroids also facilitate coping with stress and have been implicated in stress-related pathologies (1–4).

Although the most-studied steroids are produced in the periphery, some of them, named neurosteroids, are also synthesized directly in the brain (5, 6) from the putatively inactive precursor pregnenolone (3 β -hydroxypregn-5-en-20-one) (5). Active neurosteroids, such as pregnenolone sulfate (20-oxo-5-pregnen-3 β -yl sulfate), allopregnanolone (3 α -hydroxy-5 α -pregnan-20-one), and DHEA (3 β -hydroxyandrost-5-en-17-one), have been implicated in the regulation of mood and cognitive activities, and their decline has been associated with aging-related impairments (5, 7).

We investigated the involvement of neurosteroids in addiction by studying the effects of the major classes of drugs of abuse on their production in the brains of rats and mice. Concentrations of brain steroids were analyzed using gas chromatog-

raphy coupled to mass spectrometry (8, 9), which allows measuring, in the same sample, pregnenolone, DHEA, testosterone (17 β -hydroxyandrost-4-en-3-one) and its metabolite DHT (dihydrotestosterone; 17 β -hydroxy-5 α -androst-3-one), and the three stereoisomers pregnan-3-one, allopregnanolone, and epiallopregnanolone (3 β -hydroxy-5 α -pregnan-20-one). As shown for the ventral striatum (the nucleus accumbens, NAc), in the brain of Wistar rats, basal levels were approximately 1 ng per gram of tissue (ng/g) for pregnenolone and testosterone, around 0.4 ng/g for allopregnanolone and DHT, and only traces of epiallopregnanolone (<0.2 ng/g) were found (Fig. 1A). In C57BL/6N mice, the highest concentrations were found for pregnenolone and epiallopregnanolone, and the lowest concentrations were observed for testosterone. DHT was undetectable (fig. S1A). In both rat and mice brains, DHEA and pregnan-3-one were undetectable under basal conditions and after the administration of drugs.

Representative compounds of the major classes of drugs of abuse were injected into Wistar rats at doses corresponding approximately to the median effective dose (ED₅₀) for most of their unconditional behavioral effects: cocaine [20 mg per kilogram of body weight (mg/kg)], morphine (2 mg/kg), nicotine (0.4 mg/kg), alcohol (1 g/kg), and the main active principle of marijuana (*Cannabis sativa*), Δ^9 -tetrahydrocannabinol (THC) (3 mg/kg) (10). The increase in pregnenolone (Fig. 1B and table S1) induced by THC (around 1500%) was several times higher and longer-lasting (2 hours) than the one induced by the other drugs (around 300% and 30 min). Dose-response studies showed a maximal THC-induced increase in pregnenolone of approximately 3000% in both Wistar rats (Fig. 1C) and C57BL/6N mice (fig. S1).

The effects of THC on pregnenolone-derived neurosteroids were not statistically significant in mice (fig. S1). In rats (Fig. 1C), a statistically sig-

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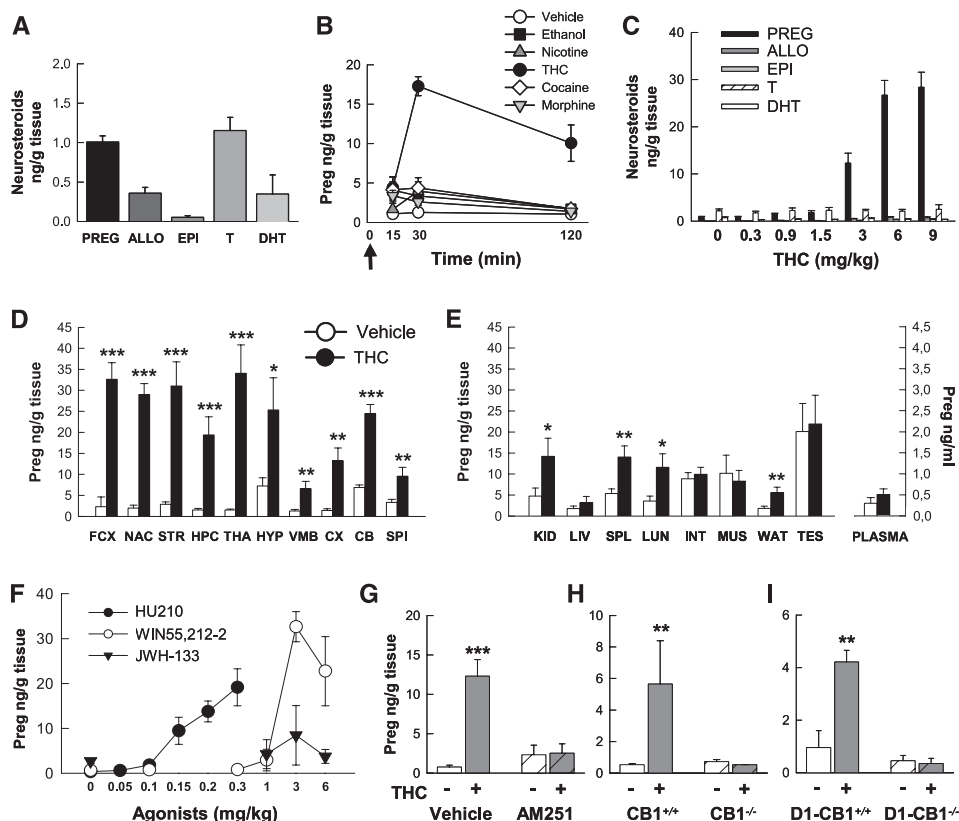
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Fig. 1. THC increases pregnenolone levels by activating the CB₁ receptor. (A) Basal levels of pregnenolone (PREG), allopregnanolone (ALLO), epiallopregnanolone (EPI), testosterone (T), and dihydrotestosterone (DHT) in the NAC.

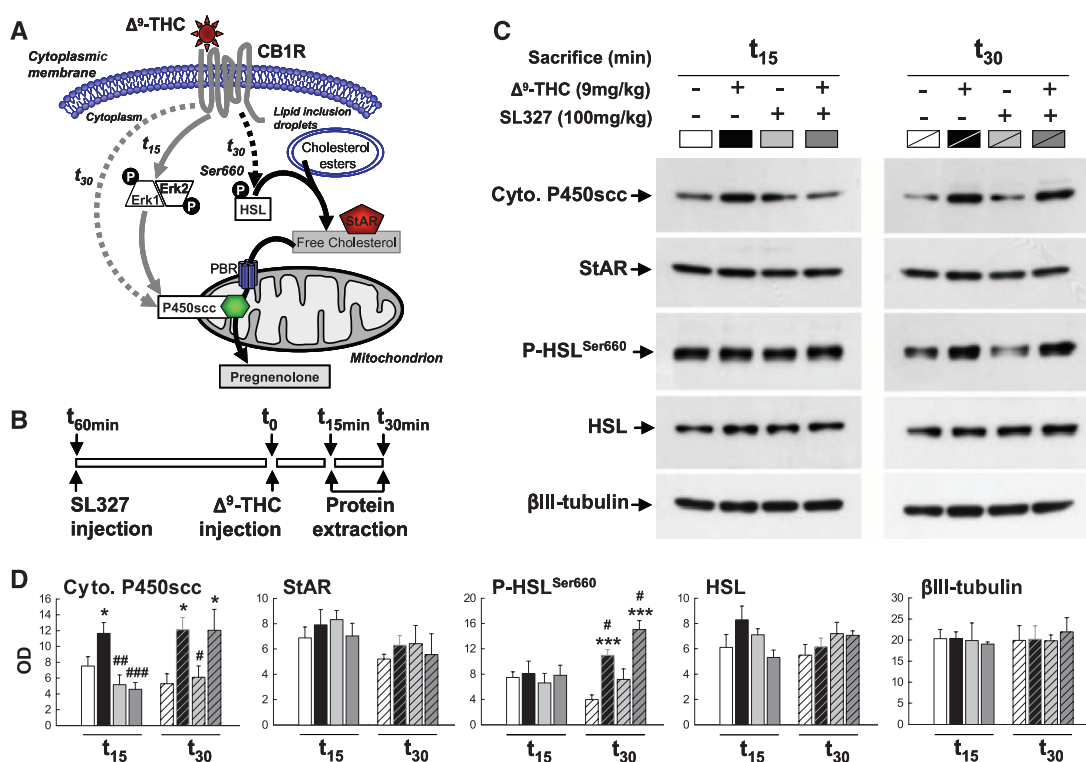
(B) Compared to the major classes of drugs of abuse, cocaine [20 mg/kg, administered intraperitoneally (ip)], morphine (2 mg/kg, ip), nicotine (0.4 mg/kg, ip), and ethanol (1 g/kg, ip), THC (3 mg/kg, ip) induced the highest increase in pregnenolone concentrations in the NAC. The arrow indicates the time of drug injection. (C) THC dose-dependently increased [$F(6,30) = 17.2$, $P < 0.001$] pregnenolone concentrations in the NAC, with minor effects on pregnenolone-derived downstream steroids. (D and E) THC at 9 mg/kg differently increased pregnenolone concentrations in brain structures and peripheral tissues: the prefrontal cortex (FCX), NAC, dorsal striatum (STR), hippocampus (HPC), thalamus (THA), hypothalamus (HYP), ventral midbrain (VMB), sensory motor cortex (CX), cerebellum (CB), spinal cord (SPI), kidney (KID), liver (LIV), spleen (SPL), lung (LUN), intestine (INT), muscle (MUS), white adipose tissue (WAT), testis (TES), and plasma. (F) In the NAC, the ip injection of the CB₁ agonists HU210 and WIN 55,212-2 dose-dependently increased pregnenolone levels [analysis of variance (ANOVA), $P < 0.001$ in all cases]. The CB₂ agonist JWH-133 had non-statistically significant effects. (G) The increase in pregnenolone concentrations induced by THC (3 mg/kg, ip) in the NAC was abolished by the CB₁ antagonist AM251 (8 mg/kg, ip) injected 30 min before THC. THC (12 mg/kg, ip) induced an increase in pregnenolone levels in the NAC of wild-type mice but not in KO mice with a (H) complete (CB₁^{-/-}) or (I) neuron-specific (D₁-CB₁^{-/-})



deletion of the CB₁ receptor. Data are expressed as mean $\pm$ SEM ($n = 6$ to 12 animals per group). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to animals that did not receive THC. All experiments except (H) and (I) were performed in Wistar rats.

Fig. 2. THC can increase pregnenolone synthesis through proteins involved in neurosteroidogenesis. Schematic representation of (A) the proposed molecular mechanism and (B) the protocol used. (C) Representative Western blots and (D) densitometric quantification of NAC expression of cytochrome P450scc, StAR, P-HSL^{Ser660}, HSL, and β III-tubulin proteins, in Wistar rats ip injected with THC (9 mg/kg) after treatment with SL327 or vehicle.

15 min after THC administration, the levels of cytochrome P450scc increased via an Erk1/2^{MAPK}-dependent mechanism. 30 min after THC administration, with an Erk1/2^{MAPK}-independent mechanism, cytochrome P450scc was still increased and HSL was activated by phosphorylation. THC administration did not modify the levels of StAR proteins. Data are expressed as mean $\pm$ SEM ($n = 5$ to 7 animals per group). OD, optical density. * $P < 0.05$, *** $P < 0.001$ in comparison to vehicle-treated rats (white and white-striped bars). # $P < 0.05$, ### $P < 0.005$, #### $P < 0.001$ in comparison to THC-treated rats (black and black-striped bars). Fisher's protected least significant difference post hoc test was used after ANOVA.



nificant effect was observed only for allopregnanolone and epiallopregnanolone. However, even at the highest dose of THC (9 mg/kg), the increase in these pregnenolone metabolites (Fig. 1C) was several times lower than the increase observed in pregnenolone (in ng/g: allopregnanolone = 0.73 ± 0.14 , epiallopregnanolone = 0.40 ± 0.08 , and pregnenolone = 28.38 ± 3.16).

The highest THC-induced increase in pregnenolone in Wistar rats (Fig. 1D) was observed in the NAc, prefrontal cortex, striatum, and thalamus and the lowest in the spinal cord, ventral midbrain, sensory motor cortex, and peripheral tissues such as the kidney, spleen, lung, and white fat (Fig. 1E). In the liver, gastrointestinal tract, muscle, testis, and plasma, THC had no significant effect.

The effects of THC in the brain are mainly mediated by the type-1 cannabinoid (CB₁) receptor (11–13). In the NAc, similarly to THC, injection of Wistar rats with the CB₁/CB₂ cannabinoid receptor agonists HU210 or WIN55,212-2 increased pregnenolone levels (Fig. 1F) at doses that are in line with their respective affinities for the CB₁ receptor (14). The CB₂-selective agonist JWH133 had no significant effect (Fig. 1F). THC effects on pregnenolone in the NAc were suppressed (i) by the CB₁-selective antagonist AM251 in Wistar rats (Fig. 1G); (ii) in constitutive CB₁ receptor knockout (KO) mice (CB₁^{−/−}, Fig. 1H), which lack CB₁ receptors in all cell types; and (iii) in conditional mutant mice (15), which lack CB₁ receptors in the majority of striatal γ-aminobutyric acid (GABAergic) spiny neurons [the ones containing the dopamine receptor D₁ (D₁-CB₁^{−/−})] (Fig. 1I) (9). The CB₁-KO mice were from mice strains in which the C57BL/6N genotype was predominant (six or seven backcrossing generations). These data indicate that THC increases pregnenolone through activation of the CB₁ receptor.

Pregnenolone is synthesized in the mitochondria from cholesterol by cytochrome P450scc (16, 17). When high levels of steroid synthesis are needed (Fig. 2A), new cholesterol is provided first by hydrolysis of cytoplasmic cholesterol esters by the hormone-sensitive lipase (HSL), activated via phosphorylation of serine 660, and then by cholesterol transport into the mitochondria by the steroidogenic acute regulatory protein (StAR) (16, 17).

In Wistar rats, StAR proteins were not modified by THC administration (9 mg/kg) at any time (Fig. 2, C and D) (9). In contrast, THC induced a rapid (15 min after THC administration) increase in the levels of P450scc (Fig. 2, C and D) that was dependent on the activity of the extracellular signal-regulated kinases 1/2 mitogen-activated protein kinase (Erk1/2^{MAPK}). This rapid increase in P450scc (9) was abolished by the selective inhibitor of Erk1/2^{MAPK} phosphorylation SL327 (100 mg/kg; Fig. 2, C and D). Later (30 min after THC administration), an Erk1/2^{MAPK}-independent mechanism sustained the increase in P450scc levels and phosphorylated HSL (Fig. 2, C and D). These coordinated modifications can contribute to the increase in pregnenolone induced by THC.

Among the behavioral and somatic CB₁ receptor-dependent effects of THC, the so-called “cannabinoid tetrad” (9, 18) is considered a prototypic signature of cannabinoid intoxication (14). The cannabinoid tetrad includes hypolocomotion, hypothermia, catalepsy, and analgesia. The administration of the inhibitor of pregnenolone synthesis aminoglutethimide (AMG, 50 mg/kg) (18) to C57BL/6N mice increased all of these behavioral and somatic effects of THC (Fig. 3, A to D). The injection of pregnenolone reversed the effects of AMG and inhibited the effects of THC but had no effect in animals that did not receive THC (Fig. 3, E and H). These data show that THC-induced production of pregnenolone exerts a negative feedback on CB₁ receptor activity.

Two well-known behavioral disturbances accompany cannabis use in humans: (i) an increase in food palatability and craving that can promote food intake (19, 20) and (ii) a decrease in memory performance (21). THC increases food intake in both satiated rats and food-deprived C57BL/6N mice (22, 23) and also impairs memory consolidation in an object-recognition task in C57BL/6N mice (24). Pregnenolone administration (2 to 6 mg/kg) blocked THC-induced food intake in Wistar rats (Fig. 4A) and in C57BL/6N mice (Fig. 4B) and blunted the memory impairment induced by THC in mice (Fig. 4C), but it did not modify these behaviors per se (Fig. 4, A to C). As previously shown (24), THC-induced memory impairments were not due to nonspecific motor effects of THC, because THC did not significantly modify locomotor activity during the object-recognition task.

Cannabinoid drugs modulate brain activity and behavior principally by the activation of presynaptic CB₁ receptors, which inhibit the release of several neurotransmitters and in particular GABA and glutamate (25). We assessed the effect of THC on glutamate release (9) by measuring excitatory postsynaptic currents (EPSCs) in NAc principal neurons in brain slices obtained from adult Sprague-Dawley rats (Fig. 4, D and E). Bath application of THC (20 μM) reliably inhibited synaptic transmission in control slices ($34.3 \pm 3.7\%$ of inhibition). The effect of THC was significantly attenuated when slices were pre-treated with pregnenolone 100 nM ($15.1 \pm 1.8\%$ of inhibition). These effects were probably due to a presynaptic action of pregnenolone. Thus, pregnenolone blocked the increase in paired-pulse ratio (PPR) induced by THC (9) but did not modify either the amplitude or the decay time of miniature EPSCs (mEPSCs) (table S2). Changes in PPR and in the mEPSC parameters studied here are indicators of changes in neurotransmitter release and in postsynaptic response, respectively.

To analyze the potential effects of pregnenolone on the development of cannabis abuse and dependence, we first studied dopamine release in the NAc (9). Activation of dopaminergic neurons and increase in NAc dopamine extracellular levels are common effects of most drugs of abuse and have been implicated in drug addiction (26).

In anesthetized Sprague-Dawley rats, we simultaneously used microdialysis and extracellular unitary recordings (9) to estimate dopamine release in the NAc and the firing activity of

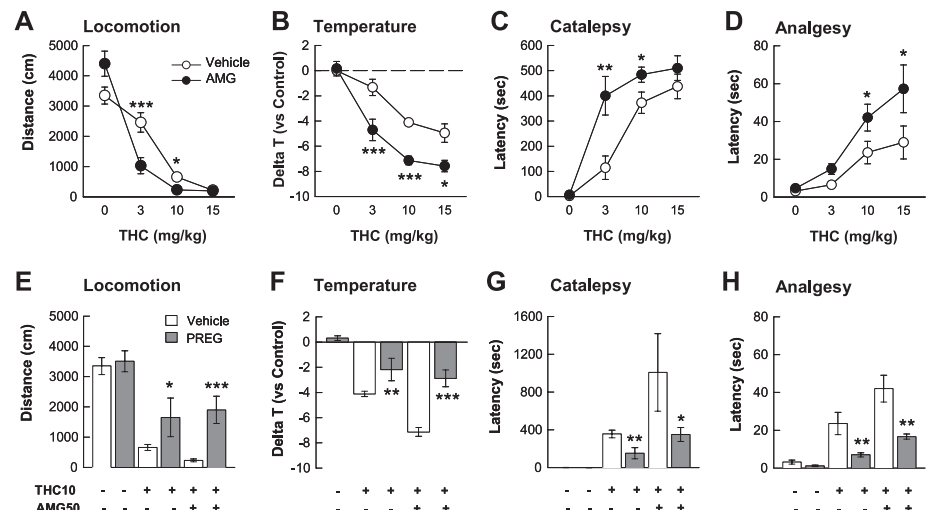


Fig. 3. The cannabinoid tetrad induced by THC was inhibited by pregnenolone. In C57BL/6N mice, the administration, 30 min before THC, of AMG (50 mg/kg, ip), a P450scc inhibitor that blocks pregnenolone synthesis, increased the effects of THC: (A) hypolocomotion [$F(3,98) = 13.8$, $P < 0.001$], (B) hypothermia [$F(3,98) = 4.7$, $P < 0.01$], (C) catalepsy [$F(3,98) = 2.1$, $P < 0.05$], and (D) analgesia [$F(3,98) = 2.2$, $P < 0.05$]. (E to H) Pregnenolone [6 mg/kg, administered subcutaneously (sc)] administered at the same time as AMG (50 mg/kg, ip) prevented the increase in the responses to THC (10 mg/kg, ip) induced by AMG. Injection of pregnenolone (6 mg/kg, sc) alone 30 min before THC (10 mg/kg, ip) also reduced the behavioral effects of THC. Pregnenolone had no effects in animals that did not receive THC. Data are expressed as mean $\pm$ SEM ($n = 6$ to 12 animals per group). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ as compared to vehicle-treated mice.

dopaminergic neurons in the ventral tegmental area (VTA), respectively. THC, administered intravenously at escalating cumulative doses (0.15, 0.3, 0.6, and 1.2 mg/kg) infused at 1-min intervals (9), induced a significant increase in extracellular NAc dopamine levels (Fig. 4G) and in the firing

activity of VTA neurons (Fig. 4F). Both effects were blunted by pretreatment with pregnenolone (2 mg/kg) (Fig. 4, F and G).

We then analyzed the impact of pregnenolone on the reinforcing effects of cannabinoid drugs, using the intravenous self-administration model

(9). In this model, CD1 mice were used, because this strain readily learns to produce an operant response (nose-poking into a hole) to obtain an intravenous infusion of CB₁ agonists. Mice readily learned to self-administer the CB₁ agonist WIN55,212-2, showing a clear preference for the device that triggered the infusion of the drug (active hole) in comparison to the inactive device, in which responding had no scheduled consequences (inactive hole) (Fig. 4H). Injections of pregnenolone (2 and 4 mg/kg) before each self-administration session reduced the intake of WIN 55,212-2 (Fig. 4I) and reduced the break point in a progressive ratio schedule (Fig. 4J), which is considered a reliable measure of the motivation for the drug (9).

To provide first insights about the mechanism of action through which pregnenolone can modify the behavioral and neurobiological effects of THC, we studied the effects of pregnenolone in cell lines expressing the human CB₁ (hCB₁) receptor (fig. S2). Briefly (9), pregnenolone (up to 100 μ M) did not modify the equilibrium binding of the radiolabeled CB₁ receptor agonists [³H]CP55,940 and [³H]WIN 55,212-2 (fig. S2A). In contrast, pregnenolone (between 10 nM and 1 μ M, depending on the cellular model) inhibited the increase in P-Erk1/2^{MAPK} and the decrease in cellular and mitochondrial respiration induced by THC (27) (fig. S2, B to F). This range of pregnenolone concentrations is compatible with the ones (between 10 and 80 ng/g, approximately 30 and 250 nM, respectively) that are observed after THC injections (Fig. 1 and fig. S1) or pregnenolone injections at behaviorally active doses (fig. S3). Pregnenolone up to 1 μ M did not decrease the THC-induced reduction of adenosine 3',5'-monophosphate (cAMP).

These effects suggest that pregnenolone acts as a signaling-specific negative allosteric modulator. Synthetic negative allosteric modulators of CB₁ receptors have been described to display signaling pathway specificity (28, 29). However, these drugs increase agonist binding affinity to the CB₁ receptor, increase agonist-induced Erk1/2^{MAPK} phosphorylation, and inhibit CB₁ agonist-induced inhibition of adenylyl cyclase (28, 29). One possible explanation of these differences is that synthetic antagonists bind to a structural pocket that is devoid of a physiological binding function. In contrast, the endogenous negative allosteric modulator pregnenolone probably binds to a different, evolution-selected, physiologic binding pocket. By using the Forced-Biased Metropolis Monte Carlo (MMC) simulated annealing program (9, 30), we found a potential binding pocket for pregnenolone in the lipid facing the TMH1/TMH7/Hx8 region of the CB₁ receptor (fig. S4A). This binding pocket was validated using a mutant hCB₁ receptor (9) that contained a point mutation that should forbid the binding of the ketone end of pregnenolone to the CB₁ receptor (fig. S4B). Pregnenolone lost its inhibitory effects on THC-induced decrease in cellular respiration in cells transfected with the mutant hCB₁ receptor (fig. S4E).

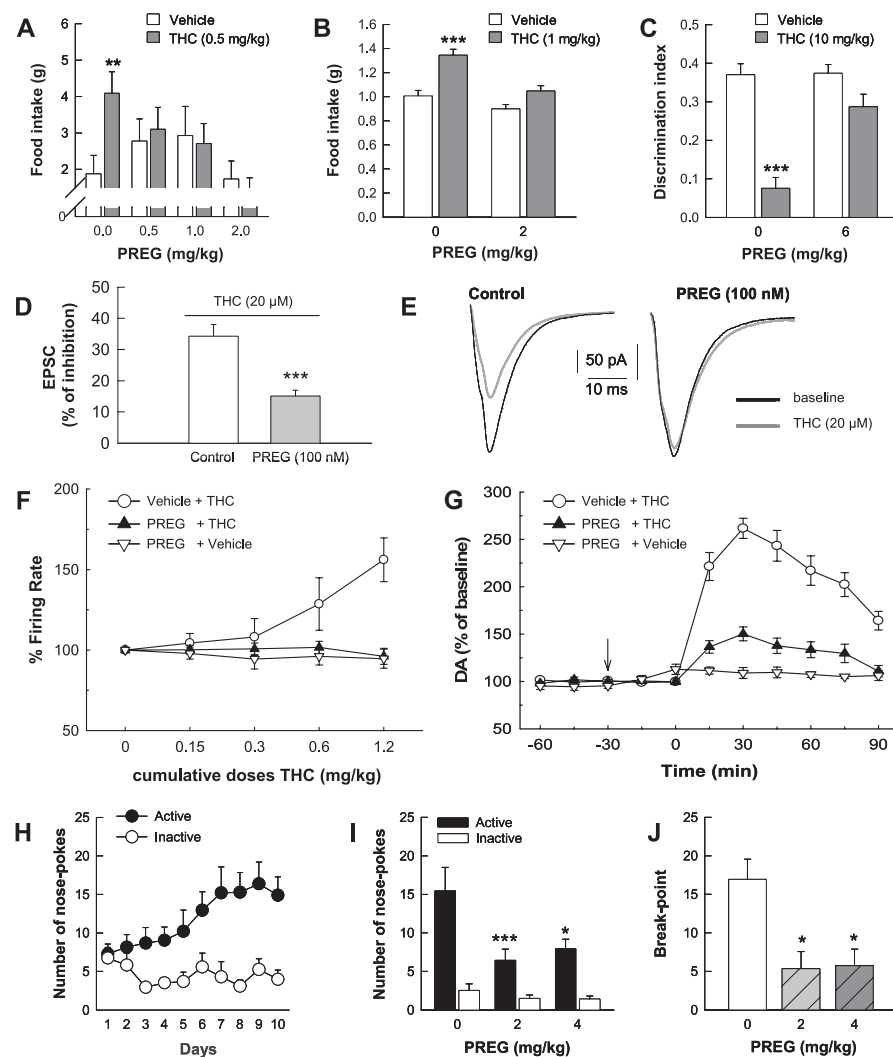


Fig. 4. Pregnenolone inhibits behavioral and neurobiological effects of cannabinoid drugs.

Pregnenolone injections inhibited the increase in food intake in (A) ad libitum fed Wistar rats [$F(3,94) = 3.65$, $P < 0.02$] and (B) 24-hour food-deprived C57BL/6N mice, as well as (C) the memory impairment [$F(3,23) = 24.6$, $P < 0.001$] induced by THC in C57BL/6N mice. (D) Bath application of THC (20 μ M) inhibited glutamatergic synaptic transmission in NAc principal neurons in brain slices obtained from adult Sprague-Dawley rats (controls, $n = 8$). This effect was reduced when brain slices were preincubated with pregnenolone 100 nM ($n = 9$). (E) Synaptic current traces from representative experiments averaged during baseline and after 40 min of THC exposure. Pregnenolone injections (2 mg/kg, sc, 30 min before THC) in Sprague-Dawley rats decreased the THC-induced increase in (F) the firing rate of VTA dopaminergic neurons [$F(4,48) = 8.33$, $P < 0.001$] and in (G) the dopamine outflow in the NAc [$F(10,120) = 20.28$, $P < 0.001$]. THC was administered intravenously at escalating cumulative doses (0.15, 0.3, 0.6, and 1.2 mg/kg) infused at 1-min intervals. (H) CD1 mice acquired intravenous self-administration of the cannabinoid agonists WIN 55,512-2 (0.0125 mg/kg per infusion) as shown by the higher number of nose pokes in the active device (hole) than in the inactive one [$F(1,18) = 38.3$, $P < 0.001$]. (I) After acquisition, the injection of pregnenolone (2 or 4 mg/kg, sc) decreased the number of responses in the active device. (J) Pregnenolone also decreased the motivation for WIN 55,512-2, as measured by the reduction in the break point in a progressive ratio schedule. Data are expressed as mean $\pm$ SEM. (A) to (C) ($n = 6$ to 12 animals per group), (F) and (G) ($n = 6$ or 7 animals per group), (H) to (J) ($n = 8$ animals per group). The arrow indicates the time of pregnenolone injection, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus vehicle-treated controls.

The results presented here provide an example of an unforeseen paracrine/autocrine loop, through which brain steroids can control the activity of a G protein-coupled receptor (GPCR). Thus, CB₁ receptor stimulation increases brain pregnenolone levels, which in turn exerts a negative feedback on the activity of the CB₁ receptor, antagonizing most of the known behavioral and somatic effects of THC. Pregnenolone probably acts as a signaling-specific negative allosteric modulator binding to a site distinct from that occupied by orthosteric ligands. Pregnenolone, similarly to some of the other previously described allosteric modulators (31, 32), does not modify agonist binding but only agonist efficacy; these effects are compatible with the allosteric two-state model (31).

Other drugs of abuse also increased pregnenolone levels, but such an increase was in a much lower range of concentrations than the ones induced by THC, which suggests a different mechanism of action. However, most drugs of abuse also modify the activity of the endocannabinoid system (33) and could increase pregnenolone through an indirect activation of the CB₁ receptor. This seemed to be the case for cocaine, whose effects on pregnenolone were blocked by pretreatment with a CB₁ antagonist (fig. S5).

Although pregnenolone has been considered an inactive precursor, our data indicate that pregnenolone, and not its downstream-derived neurosteroids, inhibits the effects of THC that are mediated by the CB₁ receptors. Thus, in mice, the administration of THC or of pregnenolone, in the range of behaviorally active doses (2 to 8 mg/kg), did not modify pregnenolone downstream-active steroids such as allopregnanolone (figs. S1 and S3). In addition, the administration of allopregnanolone did not modify behavioral responses to THC, such as THC-induced food intake (fig. S6).

An increasing number of synthetic allosteric modulators of GPCRs have been described (28, 29, 31, 32, 34, 35). However, whether endogenous allosteric modulators physiologically regulate the activity of GPCRs has been questioned (31). Recently, the lipid lipoxin A4 has been proposed as a positive allosteric modulator of CB₁ receptors, suggesting that endogenous modulation of endocannabinoid signaling is a physiological process (36). Our findings confirm and extend this hypothesis, uncovering an endogenous negative allosteric modulator of the CB₁ receptor and revealing one of the possible functions of endogenous negative allosterism: the control of GPCR overactivation.

Allosteric modulators may offer several advantages as therapeutic drugs (31, 32, 34, 35). Allosteric modulators do not modify the activity of the receptors per se but enhance or attenuate the effects of endogenous or exogenous ligands. Allosteric drugs can also be signaling-specific, thereby regulating only some of the functions of the receptor. As such, they respect the physiology of the target system, can modify only the signaling pathway involved in the disease, and have a more targeted action than orthosteric compounds (31, 32, 34, 35).

In comparison with orthosteric antagonists, drugs with the pharmacological profile of pregnenolone could have supplementary advantages for the treatment of drug dependence. When used at high doses, which effectively block the activity of the target receptor, orthosteric antagonists often induce a profound discomfort that is not well tolerated by patients. Lower doses of orthosteric antagonists are also not practical, because their reversible antagonism can be overcome by taking higher doses of the drug. Signaling pathway-specific allosteric inhibitors, such as pregnenolone, should be better tolerated because they do not produce an inhibition of all CB₁ receptor activities, and their effects cannot be overcome by increasing drug intake. This new understanding of the role of pregnenolone has the potential to generate new therapies for the treatment of cannabis dependence.

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Supplementary Materials

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Materials and Methods

Figs. S1 to S10

Tables S1 and S2

References

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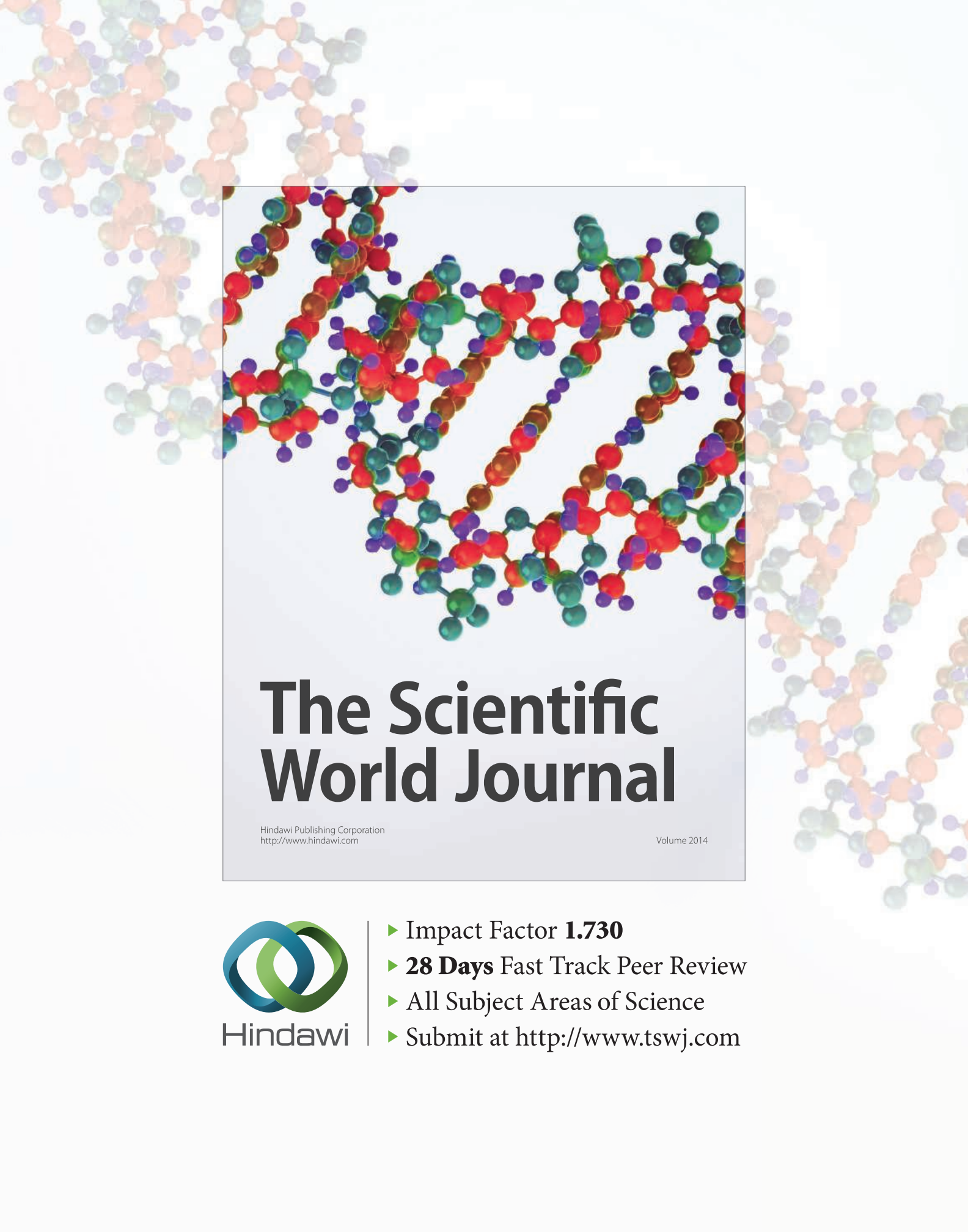
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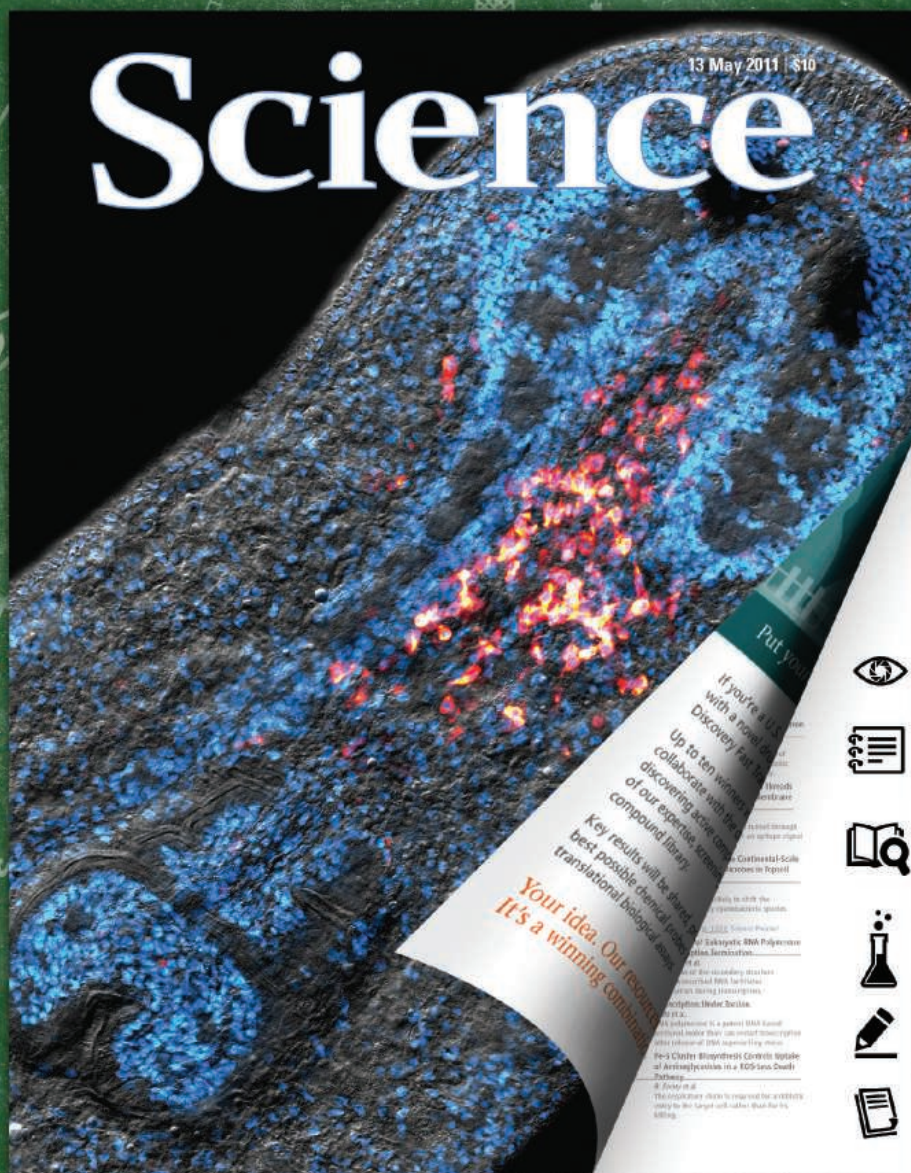
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Nominations are open from January 1 through to June 30, 2014 at 23:00 GMT.

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The Department of Energy's Oak Ridge National Laboratory has established a new distinguished research award: **The Liane B. Russell Fellowship**, named for groundbreaking female geneticist Liane Russell. Commencing 2014, this prestigious award is designed to attract a world-class and diverse workforce of early career researchers in all fields of science and engineering. Fellows will conduct high-quality research with the aim of pursuing long-term careers at ORNL, elevating the reputation of the laboratory, and becoming scientific leaders in their field. This innovative award augments ORNL's four established fellowships: the Eugene P. Wigner, Alvin M. Weinberg, Clifford G. Shull, and Alston S. Householder.

To apply for ORNL fellowships, visit www.ornl.gov/careers, select "view open positions," and search for the named fellowship. To apply directly for the Russell Fellowship, visit <http://1.usa.gov/IRu3Gk>.

UT-Battelle is recognized by our employees and the community as an inclusive environment where diversity is valued and individuals and teams are inspired to contribute fully to the organization's success. ORNL is an Equal Opportunity Employer.

DIRECTOR Arkansas Children's Nutrition Center

We are seeking to recruit a new Director of the Arkansas Children's Nutrition Center (ACNC) in Little Rock, AR. The Director will be a Professor (Tenure Track) in the Department of Pediatrics and Chief of the Division of Developmental Nutrition at the University of Arkansas for Medical Sciences (UAMS) College of Medicine.



Requirements include a PhD or MD degree and a national reputation in nutrition-related research. He/she should have demonstrated excellence in research as evidenced by accomplishments, such as a long-standing record of nationally competitive funding at the R01 level through NIH; an excellent record of publications in high quality peer-reviewed scientific journals; NIH Study Section Membership; and National Awards. The successful candidate must also have proven and effective management and interpersonal skills necessary to direct a large, interdisciplinary national human nutrition research program.

The ACNC is one of six Human Nutrition Research Centers funded by the USDA/ARS and is housed in a private research building on the campus of Arkansas Children's Hospital, which is one of the nation's largest state-of-the-art children's hospitals and the primary pediatric research and teaching site of UAMS. The position carries a competitive salary and benefits package and will remain open until February 15, 2014.

Interested parties should submit their curriculum vitae and full contact information via email to RFJ@uams.edu or mail to: **Richard Jacobs, MD, FAAP, Search Committee Chair, 13 Children's Way Slot 842, Little Rock, Arkansas 72202-3591.**

UAMS is an inclusive Equal Opportunity and Affirmative Action Employer and is committed to excellence.



南京大學

NANJING UNIVERSITY

Nanjing, CHINA

Founded in 1902, Nanjing University is one of the oldest and most prestigious institutions of higher learning in China. As a key comprehensive university with an array of outstanding faculty members, it has enjoyed coordinated development in humanities, social sciences, natural sciences, technological sciences, life sciences, modern engineering and management and so on. With the motto of "Sincerity with Aspiration, Perseverance and Integrity," Nanjing University carries the spirit of constant striving for educational and academic excellence. Today's Nanjing University invites outstanding scholars of all nationalities to join us in the mission to build this university into a world-class comprehensive research university with a global vision.

Position: Distinguished Professor

Offered by Thousand Talents Program/ Chang Jiang Scholars Program/ Deng Feng Scholar Program A

- The applicant should hold a professorship/associate professorship (or an equivalent position) in a prominent overseas university (or research institutes)
- The applicant demonstrates outstanding capabilities in scientific innovation, whose research capabilities and achievements are recognized by peers as at leading level.

Position: Young Talents Professor

Offered by Thousand Young Talents Program/Deng Feng Scholar Program B

- The applicant should hold an assistant professorship (or an equivalent position)/research fellowship in a prominent overseas university (or research institutes)
- The applicant should have been a top talent among peers and shown a strong potential to be a future leader in his/her area.

The position offers adequate scientific resources, abundant research funding, attractive salary and generous reallocation package, including the opportunity to buy an apartment in an inter price rate and subsidy.

Interested candidates please visit <http://rczp.nju.edu.cn/>



TEXAS TECH UNIVERSITY HEALTH SCIENCES CENTER™

Executive Director for the Garrison Institute on Aging

The Texas Tech University Health Sciences Center (TTUHSC) seeks an outstanding individual for the position of Executive Director of the Garrison Institute on Aging (GIA). The successful candidate is expected to have excellent scientific credentials, must have a track record of past and current National Institutes of Health or other national funding success, a substantial publication record and qualify for an academic appointment at the level of full professor. While preference will be given to individuals performing research in Alzheimer's disease and aging, all areas of research impacting neuroscience will be carefully considered and individuals in other areas of neurological investigation are strongly encouraged to apply.

A full description of the position and its requirements can be seen at <http://www.ttuhsc.edu/aging>.

Interested individuals should apply online using the following link: <https://jobs.texastech.edu/postings/58848>. A recent copy of your full Curriculum Vitae containing the names and addresses (including email address) of three individuals who will agree to act as references on your behalf should be attached to the electronic application. Screening will begin immediately and will continue until a successful candidate is selected.

Questions regarding the position can be directed to:

Douglas M. Stocco

Chair, GIA Executive Director Search Committee

Texas Tech University Health Sciences Center

Lubbock, Texas 79430.

Phone: 806-743-2505.

Email: doug.stocco@ttuhsc.edu

Texas Tech University Health Sciences Center is an Equal Opportunity/Affirmative Action Employer and actively seeks applications from women, qualified minorities and protected groups.

UConn | HEALTH CENTER

Tenure-Track Faculty Position in Computational Biology

The University of Connecticut Health Center (UCHC) is engaged in a major expansion of its research programs in computational biology, bioinformatics, and systems biology, as part of several transformative state initiatives. The Bioscience Connecticut initiative, totaling approx. \$1B, will expand UCHC health care offerings and research capabilities, including the establishment of the new Center for Quantitative Medicine (CQM). Other initiatives, totaling over \$2B, include the establishment at UCHC of the new Jackson Laboratory for Genomic Medicine (JAXGM) and the Institute for Systems Genomics, as well as the Next Generation Connecticut initiative at the main campus, expanding the overall research efforts of the University. The UCHC School of Dental Medicine (SDM) is recruiting a faculty member at the assistant or associate professor level in computational biology, who will play an important role in integrating these different initiatives, aided by a tenure track appointment in an appropriate department in the SDM, a faculty appointment at JAXGM and in CQM, and an affiliation with the Institute for Systems Genomics. This unique rich combination of research capabilities in a very dynamic and emerging environment provides this new faculty recruit with an opportunity to create a truly transformative research program.

The successful candidate will be expected to develop an externally-funded research program in computational biology with a focus on microbial genomics, metagenomics, and microbiome analysis. Opportunities exist for collaborations with SDM faculty in the areas of microbial genomics, ecology and/or host-pathogen interactions as they pertain to oral diseases. Contribution to the classroom teaching of quantitative methods within the SDM and mentoring of graduate students are also expected. It is crucial in this position to be a consummate team player in a highly interdisciplinary and dynamic environment that brings together clinicians, biologists, molecular geneticists, and quantitative scientists.

Interested applicants should apply at <https://jobs.uchc.edu/>, search code 2014-483.

For inquiries, please contact Reinhard Laubenbacher, Center for Quantitative Medicine, 860-436-7516, laubenbacher@uchc.edu

UCHC is an Equal Opportunity Employer M/F/V/PwD

Universität
Konstanz



The University of Konstanz with its »Institutional Strategy to promote Top-Level Research« has been receiving continuous funding since 2007 within the framework of the Excellence Initiative by the German Federal and State Governments.

The Faculty of Sciences, Department of Chemistry, seeks to fill a

W 3-Full Professorship of Organic Chemistry: Chemistry of Natural Products, Aromatic Compounds and Heterocycles

at the earliest possible date.

The research focus of the professorship within organic chemistry is in the area of natural products synthesis and the development of synthetic methods and should complement the research activities of the Department of Chemistry.

Further information can be obtained by contacting Prof. Dr. V. Wittmann (Valentin.Wittmann@uni-konstanz.de). Applications comprising a cover letter, curriculum vitae, publication list, a list of grants and awards, details of teaching experience, as well as statements of current research topics, future research directions and interests, copies of academic degrees (see pdf-link below on our website) should be sent electronically **until 26 January 2014 under the reference number 2013/220** formatted into one pdf-file to: Prof-2013-220@uni-konstanz.de.

Further information is available on our website:

<http://www.uni-konstanz.de/stellen> or by contacting Mr. Hanns Fahlbusch by e-mail: Hanns.Fahlbusch@uni-konstanz.de.

Director of Diabetes and Obesity Research Center

Winthrop-University Hospital is searching for the Director of its Diabetes and Obesity Research Center. The successful candidate will be an experienced M.D. or PH.D. clinical or basic scientist with a strong track record of obtaining extramural funding, leadership skills to build a nationally recognized program of research, as well as academic achievement supporting appointment at the Associate or Professor level. The Director will play a pivotal role in advancing Winthrop's diabetes-focused mission.

Winthrop-University Hospital, a 591-bed clinical campus of Stony Brook University School of Medicine, seeks to develop as a national leader in diabetes research and care. Its newly constructed 95,000 square foot Research and Academic Center will open in late 2014. The building will house basic science laboratories and a clinical research center, as well as adult and pediatric endocrine and diabetes services. The new building is designed to support translational studies and provides ample space for expansion of the research program. Currently, diabetes related research is being carried out by basic and clinical investigators in beta cell biology, renal, cardiovascular and central nervous system complications, effects of diabetes on wound healing, and therapies for obesity. Winthrop has a strong set of clinical and educational programs in diabetes and there are a growing number for collaborations between researchers and clinical faculty. Winthrop investigators also actively collaborate with scientists at other major academic centers. The development of Winthrop research is strongly supported by the Board of Trustees and a highly competitive recruitment package is available in order to expand the program.

Established in 1896, Winthrop is located in Western Nassau County on Long Island, one block from Mineola LIRR station, with easy access to Manhattan and area beaches.

Please send C.V. and cover letter to:

amjacobson@winthrop.org

Alan M. Jacobson, M.D.

Chief Research Officer

Winthrop-University Hospital

222 Station Plaza North, Suite 510

Mineola, NY 11501

An EOE m/f/d/v



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the best.



UNIVERSITÄT BASEL

Professor in Modeling of Biological Processes in Space and Time

The Biozentrum of the University of Basel in Switzerland invites applications for a professorship in modeling of spatiotemporal biological processes. The position can be filled at the Assistant (Tenure-track), Associate or Full Professor level depending on the qualifications of the applicant.

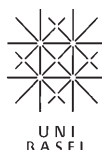
The ideal candidate should combine her/his activity in the development of novel computational and theoretical methods with a deep understanding of biology and focus on specific questions from areas in systems biology such as (but not limited to) regulatory processes, spatiotemporal patterns of gene expression, single-cell dynamics and stochastic phenomena, dynamics of chromatin, membranes, chromatin organization, morphogenesis and multiscale modeling. Although the focus of our search is on candidates at the forefront of computational and theoretical method development, applications from candidates that combine their theoretical research with wet lab approaches are also encouraged.

The candidate should have a strong research record, complementing the research portfolio of the Biozentrum, and a desire to engage in local collaborations. He/she should have very good communication skills, and is expected to actively participate in teaching at the undergraduate and postgraduate level.

The Biozentrum offers an outstanding scientific environment and an attractive research endowment. The city of Basel is the home of a vibrant international life science community and it further provides a high standard of living and a superb cultural atmosphere.

Applications, including CV, list of publications and a short research summary, should be sent by e-mail (pdf or zip) to Prof. Dr. Jörg Schibler, Dean, Faculty of Science, University of Basel, Klingelbergstrasse 50, 4056 Basel, Switzerland, to dekanat-philnat@unibas.ch. For informal inquiries please contact Prof. Dr. Erich A. Nigg (erich.nigg@unibas.ch, phone: +41-61-267 16 56).

The deadline for receipt of applications is March 15, 2014. The University of Basel is an equal opportunity employer and encourages applications from female candidates.





AAAS is here – helping scientists achieve career success.

Every month, over 400,000 students and scientists visit ScienceCareers.org in search of the information, advice, and opportunities they need to take the next step in their careers.

A complete career resource, free to the public, *Science Careers* offers a suite of tools and services developed specifically for scientists. With hundreds of career development articles, webinars and downloadable booklets filled with practical advice, a community forum providing answers to career questions, and thousands of job listings in academia, government, and industry, *Science Careers* has helped countless individuals prepare themselves for successful careers.

As a AAAS member, your dues help AAAS make this service freely available to the scientific community. If you're not a member, join us. Together we can make a difference.

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North Orange County Community College District

North Orange County
Community College District
is now hiring for
the following position:

Dean Natural Sciences

BASIC FUNCTION: Under the direction of the Chief Instructional Officer, this position is responsible for performing a variety of administrative and supervisory duties related to the functions and activities of a major multi-disciplinary instructional division within the college.

MINIMUM QUALIFICATIONS: Master's degree from a regionally accredited institution AND one year of formal training, internship, or leadership experience reasonably related to the position.

Completed District application required by date indicated. For more information and full job announcement visit : <http://apptrkr.com/422882> or call (714) 808-4810.

DEADLINE FOR APPLICATIONS: Application package must be received by 5:00 pm, February 20, 2014. Postmarks will not be honored. Application packages received after the closing deadline will not be accepted.

The North Orange County Community College District, in compliance with all applicable Federal and State laws, does not discriminate on the basis of race, color, national origin, ancestry, marital status, age, religion, disability, sex, or sexual orientation in any of its policies, procedures, or practices. The District is also committed to maintaining campuses that are free of harassment, drugs, and alcohol. A copy of the District's full policy on non-discrimination, sexual harassment, sexual assault, treatment and counseling, and maintenance of a drug-free environment is available in the District's Human Resources office.



VANDERBILT UNIVERSITY

Chair, Department of Pharmacology Vanderbilt University School of Medicine

Vanderbilt University School of Medicine announces a national search for the Chair of the Department of Pharmacology.

The Department of Pharmacology at Vanderbilt is among the most distinguished Pharmacology departments in the U.S. Its 30 primary faculty have strong sponsored research support and play prominent roles in several key institutional Centers. The Department has particular scientific strengths in chemical biology and drug discovery, signal transduction, lipid metabolism, cardiovascular biology, and neuroscience, and has a strong tradition of training future leaders in pharmacology.

The next Chair of Pharmacology will have a sustained record of excellence in scholarly activity, an exceptional scientific reputation, and will demonstrate the visionary leadership skills commensurate with the quality of the Department. He/she will work closely with related departments, will have been active in national activities, and have a professional network that will be an important asset in recruitment and reputation building.

Candidates interested in this exciting opportunity should submit a letter of interest and CV to Ian Macara, Ph.D. c/o Suzanne Alexander at suzanne.alexander@vanderbilt.edu.

*Vanderbilt University is an
Equal Opportunity, Affirmative Action Employer.*

It takes a team to save a child
...and professionals like you to help make it happen.

Faculty Positions

Department of Immunology

The Department of Immunology seeks applicants for several faculty positions at all levels (Assistant, Associate or Full) in areas of research exploring the cell biology of the immune system. St. Jude offers a remarkable opportunity to perform cutting-edge research with outstanding institutional support and exceptional core facilities in an environment of open collaboration. Successful applicants will present a vibrant research program with potential for interaction within the department and institution. All areas of immunology are welcome, but preference will be given to those areas that shed light on basic cellular and molecular processes within the context of the immune system.

Interested applicants should send CV, a letter of research interests and contact information for three references to:

Douglas R. Green
Chair, Department of Immunology
St. Jude Children's Research Hospital
332 N. Lauderdale Street, Memphis, TN 38109
Douglas.Green@stjude.org

To learn more, visit
www.stjude.org/jobs

St. Jude Children's Research Hospital is one of the premier pediatric cancer hospitals in the United States and has a large faculty of clinical and basic investigators organized into traditional academic departments. Strong interdepartmental programs are present in Hematological Malignancies, Solid Tumor Biology, Neuro-oncology, Molecular Oncogenesis, Transplantation and Gene Therapy. Clinical and basic research activities are carried out in adjoining state-of-the-art buildings that provide an environment that fosters close collaboration between clinical and basic investigators.

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Faculty Positions in

Department of Biochemistry and Molecular Biology

The Department of Biochemistry and Molecular Biology at LSU Health Sciences Center – Shreveport is seeking academic scientists to fill tenured or tenure-track faculty positions at the rank of **ASSISTANT, ASSOCIATE, or FULL PROFESSOR**. Requirements include a PhD and/or MD in Biochemistry, Molecular Biology, and/or a related discipline, and at least five years of postdoctoral research experience. Successful applicants must have a record of scholarly contributions commensurate with respect to rank, and a track record of extramural funding. Applicants will be expected to maintain extramural funding, participate in both medical and graduate school courses, and support and mentor graduate students and postdoctoral trainees. Current faculty are engaged in research in the areas of cancer biology, neurosciences, diabetes, urosciences, and yeast genetics. Ongoing research projects in the department are focused on studies related to DNA damage and repair; epigenetic mechanisms regulating transcription; translational and post-translational regulation of protein synthesis and degradation; cell signaling; metastasis suppressor genes; developmental therapeutics; enzyme catalysis; and molecular chaperones.

Applicants should submit their curriculum vitae, summary of current research and support, outline of future research plans, and contact information for three references. The search committee will request letters from references as needed. Please e-mail PDF files to Prof. Hari K. Koul, Carroll W. Feist Endowed Chair, Professor and Chairman, Department of Biochemistry and Molecular Biology, and Director of Basic and Translational Research - Feist-Weiller Cancer Center, attention of **Ms. Carlotta Ford** at cford5@lsuhsc.edu. Complete applications will be reviewed on a rolling basis until the positions are filled. Additional information is available at (<http://www.lsuhseshreveport.edu>) and (www.shrevebiochem.com).

LSUHSC is an Equal Opportunity/Affirmative Action Employer.



Medical College
of Georgia

VASCULAR BIOLOGY CENTER



ASSISTANT/ASSOCIATE/FULL PROFESSOR TENURE-TRACK

Applications are invited from qualified applicants for available tenure-eligible faculty positions at all ranks.

The Vascular Biology Center of the Georgia Regents University presently includes 15 tenured/tenure-track core faculty, 78 staff (research faculty, Pre- and Post-doctoral Fellows and Research technicians) in recently-renovated, well-equipped laboratory space (20,000+ square feet). Desirable applicants will complement the highly-collaborative faculty of the VBC and contribute to the Center's extramural grant portfolio (currently >\$8M) as well as its graduate and post-doctoral training programs. Excellent core facilities exist within the Center (including access to Vevo 2100, Seahorse XF and metabolic phenotyping cores) and within the institution (small-animal imaging, biotelemetry, microarray, deep-sequencing and confocal imaging).

The successful applicants will have excellent communication skills and research interests in vascular biology or related conditions (i.e. obesity/diabetes, heart disease). Associate/Full Professors should have established NIH-funded programs. Preference will be given to applicants with interests complementing those of existing faculty as described at <http://www.gru.edu/centers/vbc/>. Highly competitive salaries and excellent start-up packages are available. Inquiries and applications (including full CV, letter of research interests/career plans) should be addressed to Dr. David Stepp (dstepp@gru.edu) and Dr. Neal Weintraub (nweintraub@gru.edu).

Georgia Regents University is an Equal Opportunity and Equal Access Institution, AA/EEO/Equal Access or AA/EEO/Equal Access/ADA Employer. Applications from women and under represented minorities are particularly encouraged. On-Line Application <http://www.gru.edu/facultyjobs>, reference #6179, Vascular Biology Center.

POSITIONS OPEN

Yale

ELECTROPHYSIOLOGIST/BIOPHYSICIST Channels and Hyperexcitability Disorders

The Center for Neuroscience & Regeneration Research at Yale University has an opening for a channel physiologist, to join multidisciplinary research group studying properties and roles of ion channels associated with hyperexcitability disorders. Doctoral degree and substantive experience at channel physiology are essential. Superb opportunity to work collaboratively within a multidisciplinary group with molecular and cell biologists, pharmacologists, pain researchers, biophysicists and physiologists using voltage-clamp, current-clamp, dynamic clamp, and optical methods to study ion channels associated with hyperexcitability disorders. See Dib-Hajj *et al.*, *Nat Rev Neurosci*, **14**(1): 49-62, 2013; Yang *et al.*, *Nature Comm.*, **3**: 1186, 2012. Send curriculum vitae, three letters of reference, and statement of interests to e-mail: stephen.waxman@yale.edu. Women and members of underrepresented minority groups are encouraged to apply. Affirmative Action/Equal Opportunity Employer.

TENURE-TRACK FACULTY Neurobiology and Behavior

The Department of Neurobiology and Behavior at Stony Brook University is recruiting several tenure-track faculty members. Applicants must have a Ph.D., M.D., or equivalent degree, and at least two years of postdoctoral experience. Outstanding candidates in all fields of systems neuroscience will be considered. Candidates may also be considered for the new cross-disciplinary Stony Brook Cluster Initiative in the Neuroscience of Anxiety and Depression.

Exceptional packages include state-funded salary and benefits, newly renovated laboratory space and generous startup funding. Successful candidates will join an interactive, diverse group at Stony Brook University and its affiliated institutions, and will actively contribute to the Department's programs in undergraduate, graduate, and medical education.

Interested individuals should send curriculum vitae, a statement of research interests, and contact information for three people from whom letters of reference may be requested to Systems Neurobiology Search Stony Brook University at Academic Jobs Online at website: <https://academicjobsonline.org/ajo/jobs/3544>. Electronic submissions are strongly preferred. Review of applications will begin January 18, 2014 and will continue until the available positions are filled.

To view application procedure, full position description or to apply online visit website: <http://www.stonybrook.edu/jobs> (Ref. # F-8357-13-12).

Affirmative Action/Equal Opportunity Employer.

ASSISTANT PROFESSOR in Synthetic Biology

Missouri University of Science and Technology

The Department of Biological Sciences at the Missouri University of Science and Technology (Missouri S&T) seeks to fill a tenure-track faculty position at the Assistant Professor level. We are seeking a candidate who uses Synthetic Biology in an interdisciplinary approach to design and engineer cells to address fundamental questions in biology. We expect the faculty member will collaborate with colleagues in departments and centers on campus involved in interdisciplinary biology research. The Department has 10 full-time faculty and awards B.A., B.S., and M.S. degrees in Biological Sciences. Opportunities for interdisciplinary research abound and such activities are strongly encouraged with several campus-wide research centers. Applicants may apply online at website: <http://jobs.mst.edu>. Please include Reference Number 00059853. Applications should be submitted by January 31, 2014 to receive full consideration.

POSITIONS OPEN

ASSISTANT PROFESSOR in Landscape Ecology

The University of Arkansas Department of Biological Sciences seeks applicants with exceptionally strong computational and modeling skills for a tenure-track Assistant Professor position in Landscape Ecology (Position Y14961, website: <http://hr.uark.edu/jobdetails.asp?ListingID=7075>). Requirements: Ph.D., postdoctoral experience in ecology, and strong research record. Expectations: establish externally funded research program, contribute to undergraduate-graduate education, and professional service. Submit complete application (curriculum vitae, research and teaching statements, and three reference letters) as PDF or paper application to Dr. Michelle Evans-White (e-mail: ecology@uark.edu) by 30 January 2014. Review will continue until position is filled. The University of Arkansas is an Equal Opportunity/Affirmative Action Institution. All applicants are subject to public disclosure under the Arkansas Freedom of Information Act and persons hired must have proof of legal authority to work in the United States.

The Center for Genomics and Bioinformatics (CGB) at Indiana University (website: <http://cgb.indiana.edu>) is currently accepting applications for two positions in the Center's next-generation sequencing (NGS) operation: a **LABORATORY MANAGER**, responsible for NGS library construction, generating methods for robotic automation, and catalyzing interactions between the CGB and IU faculty; and a **LABORATORY TECHNICIAN**, whose primary responsibility will be library construction (both manual and robotic). Both positions require excellent communication skills; experience in NGS library construction and familiarity with laboratory automation is preferred. The positions require a Master's degree (laboratory manager) or Bachelor's degree (laboratory technician) in a related field.

The positions are available immediately. Applications received by February 1, 2014 will be assured full consideration. Applicants should review the application requirements and submit their application at Laboratory Manager website: <http://indiana.peopleadmin.com/postings/702> and Laboratory Technician website: <http://indiana.peopleadmin.com/postings/703>.

Enquiries should be directed to: Scott Michaels, Director, Center for Genomics and Bioinformatics, Jordan Hall 044, 1001 E 3rd St, Bloomington IN 47405, or e-mail: jobs@cgb.indiana.edu.

Indiana University is an Affirmative Action/Equal Opportunity Employer.

ASSISTANT PROFESSORS Fudan University, China Department of Chemistry

Fudan University seeks applicants at the Assistant Professor (tenure track) level for faculty positions in the Department of Chemistry. Of particular interest are persons with background in biochemistry, inorganic chemistry, and organic chemistry. However, creative and energetic individuals who show extraordinary promise or accomplishment in any area will be considered. We require a Ph.D. in Chemistry or a closely related discipline, and postdoctoral experience is desirable. Fudan University, located in the city of Shanghai, is one of the most prestigious universities in China. The positions include excellent startup package and comprehensive benefits. To apply, please send curriculum vitae, detailed statements of research, and names and addresses of three references to e-mail: chemhr@fudan.edu.cn. Major startup funding will be from the China "National 1000 Plan" (Youth Program, website: <http://www.1000plan.org>), and the application to the program will be submitted with Fudan University as the host University.

POSITIONS OPEN

VISITING ASSISTANT PROFESSOR of Biology (Molecular Biology)

Kenyon College invites applications for a one-year Visiting Assistant Professor in Biology (Molecular Biology). Teaching will include molecular biology laboratory courses, and introductory biology lecture and labs, with opportunities for mentoring undergraduate research. Candidates should hold a Ph.D. degree and demonstrate teaching excellence. To apply, visit the online application site: website: <http://employment2.kenyon.edu/postings/1657>. Review of applications will begin February 3, 2014 and will continue until the position is filled. As an Equal Opportunity Employer, Kenyon is committed to building a diverse faculty and encourages the applications of women and minority candidates.

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experts.

www.sciencecareers.org

- Job Postings
- Job Alerts
- Resume/CV Database
- Career Advice
- Career Forum

Science Careers

From the journal Science



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scientists agree—
we are the most
useful website.

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